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Evaluation of neuromuscular transmission in organophosphorus pesticide toxicity

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Declaration

I hereby declare that the work contained within, and the composition of, this thesis is my own and has not been submitted for any other degree.

Kosala Nimanthi Dissanayake

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Abstract

Organophosphorus (OP) pesticide toxicity is a global health problem. Respiratory failure due to neuromuscular transmission dysfunction accounts for about 300,000 deaths annually in rural Asia. However, the clinical manifestation is complex, and described in terms of acute, intermediate, and chronic syndromes. The underlying mechanism of toxicity is still unclear. OP pesticides contain inhibitors of acetylcholinesterase (AChE), for example dimethoate, emulsified in an organic solvent, typically cyclohexanone. A hypothesized mechanism is initial excitotoxicity through inhibition of acetylcholinesterase followed by failure of neuromuscular synaptic transmission. I tested this electrophysiologically *in vitro* by measuring properties of spontaneous miniature endplate potentials (MEPPs) and evoked endplate potentials (EPPs) in isolated sciatic nerve/flexor digitorum brevis muscles from mice, bathed in HEPES-buffered mammalian physiological saline (MPS). Muscle action potentials were abolished with μ-conotoxin (2μM). First, we tested the effects of plasma taken from Göttingen minipigs instilled orally (isofluorane anaesthesia) with a formulated pesticide (2.5ml/kg) whose active ingredient is dimethoate dissolved in cyclohexanone. This plasma abolished evoked synaptic transmission and increased spontaneous MEPP frequency within 60-180 minutes of bath application. However plasma from minipigs instilled with dimethoate alone produced no failure of transmission. Plasma contained either pesticide or dimethoate significantly increased the half decay time of EPPs. However, pesticide-plasma also contained the metabolites omethoate (100μM) and cyclohexanol (5 mM). We found that bath application of omethoate alone caused a potent dose-dependent increase in EPP decay time. Cyclohexanol (5 mM) also increased EPP decay time but it also decreased both the excitability of axons and MEPP amplitude. In combination, omethoate and cyclohexanol produced greater disruption of neuromuscular transmission than either dimethoate or cyclohexanone, alone or in combination and this was particularly evident in isometric tension recordings, in which prolonged after-contraction and slow relaxation were observed during and immediately following tetanic stiumuation in the presence of omethoate and cyclohexanol. Voltage-clamp recordings of endplate currents (EPC) partially supported the EPP observations. Surprisingly, cyclohexanol-treated preparations showed no significant increase in EPC and MEPC decay time. However, there was some evidence of activity-dependent decline in MEPC amplitude in cyclohexanol while quantal content in these preparations showed evidence of an increase suggesting a homeostatic response in evoked transmitter release with cyclohexanol treatment. Analysis of presynaptic currents in

cyclohexanol treated preparations also revealed preliminary evidence of sensitivity to cyclohexanol compared to control preparations. Finally, I tested the effects NMJ transmission of 24hr exposure to OP pesticide and its metabolites using a novel organ culture system, utilising a mouse mutant (Wld^S) with a slow nerve degeneration phenotype. After incubation of 24 hrs with MPS + pesticides and metabolites, these muscles showed significant reduction in function (response to nerve stimuli with EPP/action potential ± MEPPs) compared to control cultures. Together, the data indicate that failure of neuromuscular transmission by pesticide-plasma cannot be explained solely by dimethoate-mediated inhibition of acetylcholinesterase. Rather, a combination of metabolic breakdown products exerts potent, harmful presynaptic and postsynaptic effects. Either blocking the metabolic conversion of the constituents of OP pesticides, or transiently blocking their effects on receptors may therefore be an effective strategy for treatment of OP pesticide toxicity.

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Abbreviations

ACh	Acetylcholine
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
ANOVA	Analysis of variance
ChAT	Choline acetyltransferase
CH-ol	Cyclohexanol
CH-one	Cyclohexanone
CMAP	Compound muscle action potentials
CNS	Central nervous system
CTX	Conotoxin
EC	Emulsifiable concentrate
Ec 50	Effective concentration 50
EDL	Extensor digitorum longus
EPC	Endplate current
EPP	Endplate potential
FDB	Flexor digitorum brevis
IMS	Intermediate syndrome
LD 50	50% lethal dose
MEPC	Miniature endplate currents
MEPP	Miniature endplate potential
MPS	Mammalian physiological saline

nAChR	Nicotinic Acetylcholine receptor
NMJ	Neuromuscular junction
OP	Organophosphorus
OPICN	Organophosphorus induced chronic neurotoxicity
OPIDP	Organophosphorus induced polyneuropathy
PNS	Peripheral nervous system
QC	Quantal content
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF associated protein 25
SR	Sarcoplasmic reticulum
TOF	Trains-of-four
TRITC-α-BTX	Tetramethylrhodamine-isothiocyanate (TRITC) α- bungarotoxin
TS	Trangularis sterni
T50	Half decay time
***Wld*^{S}**	Wallerian degeneration slow
WHO	World Health Organization
YFP	Yellow fluorescent protein

Chapter 01: General introduction

Respiratory failure in organophosphorus (OP) pesticide toxicity accounts for thousands of deaths annually across the globe. There are two types of respiratory failure that occur during the course of OP toxicity: a) acute respiratory failure (during the early stage of pesticide poisoning) mostly due to central respiratory depression, respiratory muscle weakness and / or direct pulmonary effects such as bronchospasms and bronchorrhoes, b) delayed neuromuscular weakness and respiratory failure (24 – 72hrs after pesticide poisoning). Even though the acute respiratory failure is well characterized, the mechanisms of delayed respiratory failure are yet to be established even after several decades since its initial identification. The understanding of delayed neuromuscular weakness and respiratory failure will benefit the search for novel therapeutic treatments and thereby potentially saving tens of thousands of human lives in the future.

1.1 Pesticide toxicity

1.1.1 Importance of the problem

Pesticide toxicity can result from accidental inhalation, ingestion or though skin contamination with the product. However, deliberate, self-inflicted poisoning (e.g. attempted suicides) contributes to most of the reported cases worldwide. For instance pesticide self-poisoning leads to 250,000 – 370,000 mortalities every year, and is responsible for about one third of global suicides (Gunnell et al., 2007). Among all the pesticide-induced cases, organophosphorus (OP) poisoning is the most common in rural Asia (Gunnell et al., 2007), accounting for two thirds of the total cases (Eddleston, 2000). For example, in Sri Lanka, there were about 17,000 admissions annually in mid 1990's due to pesticide poisoning, which results in occupancy of 35% of the intensive care unit beds in the rural hospitals (Eddleston et al., 1998). In 2006, the World Health Organization (WHO) declared, that "Pesticide poisoning is the single most important means of suicide worldwide" (Bertolote et al., 2006).

1.1.2 Types of Pesticides

Many types of pesticides are widely in use world wide. These include organophosphates (for example dimethoate, chlorpyrifos), carbamates (carbofuran, propoxur), organochlorines (endosulfan, DDT), pyrethroid (etofenprox), herbicides (paraquate, proponil) and fungicides (edifenphos).

OP pesticides are widely used in agriculture and in household applications. Commercially available OP pesticides are manufactured as many formulation types including dry spray-able (e.g. wettable powders, water dispersible granule), liquid spray-able (e.g. soluble concentrate, suspension concentrate, emulsifiable concentrate, ect) and dry spreadable granules (e.g. soil applied granule on inert or fertilizer carrier) (Gouge, 2000). Emulsifiable concentrate (EC), which is a mixer of active ingredient (OP), solvent and a surfactant. Formulation improves the properties of a chemical for handling, storage, application and may substantially influence effectiveness and safety (Gouge, 2000).

According to their toxicity (active ingredient compound and its formulations) pesticides are classified into five different groups (WHO, 2005), Ia, Ib, II, III and U graded from extremely hazardous to unlikely to present acute hazard.

However, these compounds show major differences in human toxicity and fatalities within same pesticide class and with the similar agricultural indication (Dawson et al., 2010). A prospective cohort study carried out in Sri Lanka from 2002 until 2008 indicated that anticholinesterase (dimethoate, fenthion) and herbicide paraquat pesticides belonging to toxicity class II were responsible for most of the hospital administration and the mortalities.

1.1.3 Clinical manifestation (Signs and symptoms: acute, intermediate syndrome, polyneuropathy)

OP toxicity is mainly categorized as either acute or chronic, according to the onset, clinical manifestation and postulated mechanism of action. Acute toxicity is further subcategorized into acute cholinergic syndrome and “intermediate syndrome” (IMS). The onset of clinical symptoms varies depending on the route of exposure, degree of exposure and the type of OP compounds, e.g. ingestion of a large amount of oxon OP (e.g. monochrotophos) can cause symptoms within 5 minutes. By contrast, ingestion of the same amount of thiophosphate OP (fenthion) may take hours to produce clinical symptoms and signs.

Since OP pesticides inhibit the enzyme acetylcholinesterase (AChE), the effects of OP ingestion are thought to increase levels of acetylcholine (ACh) neurotransmitter in the synaptic cleft, thereby causing repeated activation of the ACh receptors. So, acute toxicity most likely arises from the overstimulation of ACh receptors present in the central and autonomic nervous systems, and at neuromuscular junctions (NMJ) in skeletal muscles.

The most common clinical signs of acute cholinergic syndrome include pinpoint pupils, muscle fasciculation, diaphoresis, emesis, diarrhoea, salivation, lacrimation and urinary incontinence connected with autonomic effect of anticholinesterase. Most deaths occur within hours of pesticide ingestion during this "cholinergic crisis" (Lotti, 2001), as a consequence of acute respiratory failure due to central respiratory depression, respiratory muscle weakness, and/or direct pulmonary effect (bronchospasm and bronchorrhoea). Other signs of excessive activity of ACh in the autonomic nervous system include muscarinic (salivation, lacrimation, urination and defecation) and nicotinic effects (fasciculation or weakness in skeletal muscle or sometimes paralysis). Restlessness, anxiety and unconsciousness are some of the findings related to effects of OP on the central nervous system (CNS). Hypoxia, hypovolemia and hypotension are some of the cardiovascular clinical manifestations. Excessive cholinergic stimulation at skeletal muscles results in fasciculation or weakness.

Intermediate syndrome is characterized by delayed muscle weakness resulting in respiratory failure without cholinergic features or fasciculation. Development of proximal muscle weakness, cranial nerve palsies, and respiratory failure lasting up to several weeks are the common clinical features of IMS. Patients need long term artificial ventilation until they regain capability of self-breathing. As a result of long term artificial ventilation during IMS, pesticide poisoning became responsible for half of the intensive care unit bed occupancy in rural Asian hospitals, leaving less opportunity to treat more debilitating diseases such as myocardial infarction, encephalitis, etc. (Eddleston et al., 1998).

IMS was first described as a syndrome of muscular paralysis occurring in conscious patients 24-96 hours following ingestion of OP pesticides, after the acute cholinergic syndrome has been treated with atropine (Senanayake and Karalliedde, 1987) .

Early studies described the involvement of central respiratory failure, bronchoconstriction and neuromuscular block in respiratory failure with anticholinesterases (De Candole et al., 1953). Wadia et al (1974) described neurological manifestation in two distinct categories: type I (present on admission and respond to atropine therapy), and type II (appearing after the commencement of treatment and not influenced by atropine). Despite the two distinct and non-overlapping syndromes of OP-induced respiratory failure suggested in the literature, many clinical cases do not directly fall in two categories (Eddleston et al., 2006). It appears that there are two distinct pathophysiologies for the onset of both types of respiratory failures

(central and peripheral); however, further research is required to identify the underlying mechanism of action.

Dysfunction in NMJ transmission affects the diaphragm and intercostal muscle, which ultimately leads to respiratory failure. Clinical and experimental studies of IMS have basically focused on structural (OP induced myopathy) and functional (electrophysiological) aspects of the NMJ. Surprisingly, however, even though this syndrome has been well documented for decades, the underlying mechanism of the neuromuscular dysfunction is still unknown. The hypothesized mechanisms of action for IMS include: overstimulation of nicotinic acetylcholine receptors (nAChRs); down regulation of post synaptic nAChRs; and nAChR conformational changes with prolonged depolarization (Senanayake and Karalliedde, 1987; De Bleecker, 1995) . Factors affecting the onset of IMS include rapidity and quantity of AChE inhibition and insufficient oxime (antidote for activating AChE enzyme)) therapy. The occurrence of IMS is also more common with certain OP pesticides, eg: parathion, methylparathion, and fenthion. Understanding distinctive electrophysiological abnormalities with nerve conduction studies (Besser et al., 1989b) facilitates predicting the development of IMS (Jayawardane et al., 2008).

Further consequences of organophosphorus toxicity include organophosphorus induced polyneuropathy (OPIDP) resulting from inhibition of neuropathy target esterase (lysophospholipase) present in nervous tissue. This occurs as a consequence of chronic OP exposure over days or weeks following acute exposure. Transient loss of neuropathic target esterase activity, putative disruption of membrane phospholipid homeostasis, axonal transport, and glial-axonal interactions are some of the mechanisms involved with OPIDP (Glynn, 2006). Indistinct distal muscle weakness, pain and progressive paralysis are the common clinical signs present in the OPIDP. There are also reports of a chronic clinical syndrome which differs from both acute toxicity and OPIDP. This involves neural degeneration and subsequent neurological, neurobehavioral and neuropsychological consequences. This syndrome is referred to as organophosphorus induced chronic neurotoxicity (OPICN) (Abou-Donia, 2003). It is thought to occur from exposure to a single, large toxic dose or to small subclinical doses of OP compounds, which later then develop as long term neurological deficit, lasting for years after exposure (Abou-Donia, 2003). One hypothesised mechanism for OPICN is either via necrotic death of brain neuronal cells or through delayed apoptotic cell death (Abou-Donia, 2003).

1.1.4 Clinical management of organophosphorus pesticide toxicity

Acute cholinergic syndrome occurs after exposure to a high dose of OP compounds. The effect of this is consistent with excessive stimulation of muscarinic and nicotinic cholinergic receptors due to persistent levels of acetylcholine (ACh) in the central, autonomic and peripheral nervous system (see above).

Diagnosis with history of acute exposure to OP is mainly from the clinical signs of cholinergic crisis. The main laboratory tests that aid diagnosis of OP poisoning are those that measure the level of specific OPs and metabolites in biological tissues and acetylcholinesterase activity in plasma or blood. Electromyography is also used as a diagnostic test for AChE inhibition at the NMJ (Jayawardane et al., 2008).

Preliminary clinical management for patients with OP toxicity are to ensure an adequate airway and ventilation (endotracheal intubation and positive pressure ventilation) and stabilization of cardiorespiratory function by reversing excessive muscarinic effects (anti-muscarinic therapy; atropine) along with the supportive care (Eddleston et al., 2004; Eddleston et al., 2008). The oximes such as pralidoxime chloride (2-PAM) or obidoxime are used as antidotes which enhances the hydrolytic regeneration of the AChE (Eyer, 2003). In some instances, evacuation of stomach content is performed by gastric lavage especially patients who have not experienced emesis after ingestion of OP (Eddleston and Clark, 2010).

1.1.5 Pesticide pharmacodynamics and clearance

The chemical nature of OP compounds is highly variable: e.g. the octanol-water partitioning value ($log\ K_{ow}$) ranges from -0.2 (monocrotophos) to 6 (bromophos ethyl) and water solubility (log_{sol}) ranges from -1.1 (prothiofos) to 6 (dicrotophos). The variable nature of these chemicals is reflected in the differing toxicity, kinetics, clinical syndrome and response to antidote.

OP pesticides are either direct (oxon) or indirect (thion) inhibitors of carboxylic ester hydrolases (e.g: acetylcholinesterase [AChE]), and butyrylcholinesterase (Kwong, 2002; Eddleston and Clark, 2010).

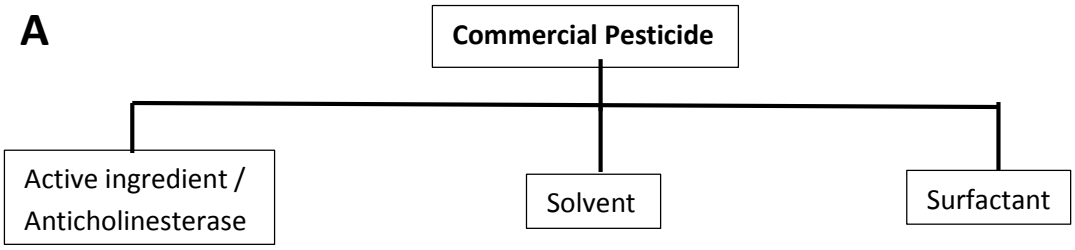
Formulation of pesticides uses solvents including organic and partially chlorinated compounds (Petrelli et al., 1993). The most common solvents used in organophosphorus pesticides are cyclohexanone and xylene.

The majority of the OP compounds are lipophilic and not ionised, therefore absorbed rapidly following ingestion. Absorption is also facilitated by the presence of solvent and emulsifier used in the technical formulation of pesticides (Vale, 1998; Kwong, 2002). Following ingestion and uptake from the gut, OP compounds accumulate rapidly in fat, liver, kidney and salivary glands (Vale, 1998; Kwong, 2002). Phosphorothioate (P=S) compounds are more lipophilic compared to phosphates (P=O), therefore stored extensively in the fat. These lipophilic compounds also cross the blood brain barrier in most cases (Vale, 1998). Inactive thiophosphorus OPs (eg: dimethoate, parathion) undergo bioactivation (desulfation) via liver cytochrome P450, producing active, oxon metabolites (eg: omethoate, paraoxon) (Foxenberg et al., 2007). Some studies have reported involvement of other enzyme complexes in the process of desulfation such as flavine-containing mono-oxygenase enzyme (Hodgson and Levi, 1992) and of n-oxidation and s-oxidation (Mileson et al., 1998). Furthermore cytochrome P450 is also involved in detoxification of both parent compound and disulphide compound through a dearylation reaction (Mutch et al., 2003; Poet et al., 2003). The main elimination pathway of these OP metabolites is via urine, while lesser amounts excrete through faeces and expired air (Vale, 1998) (Fig 1.1).

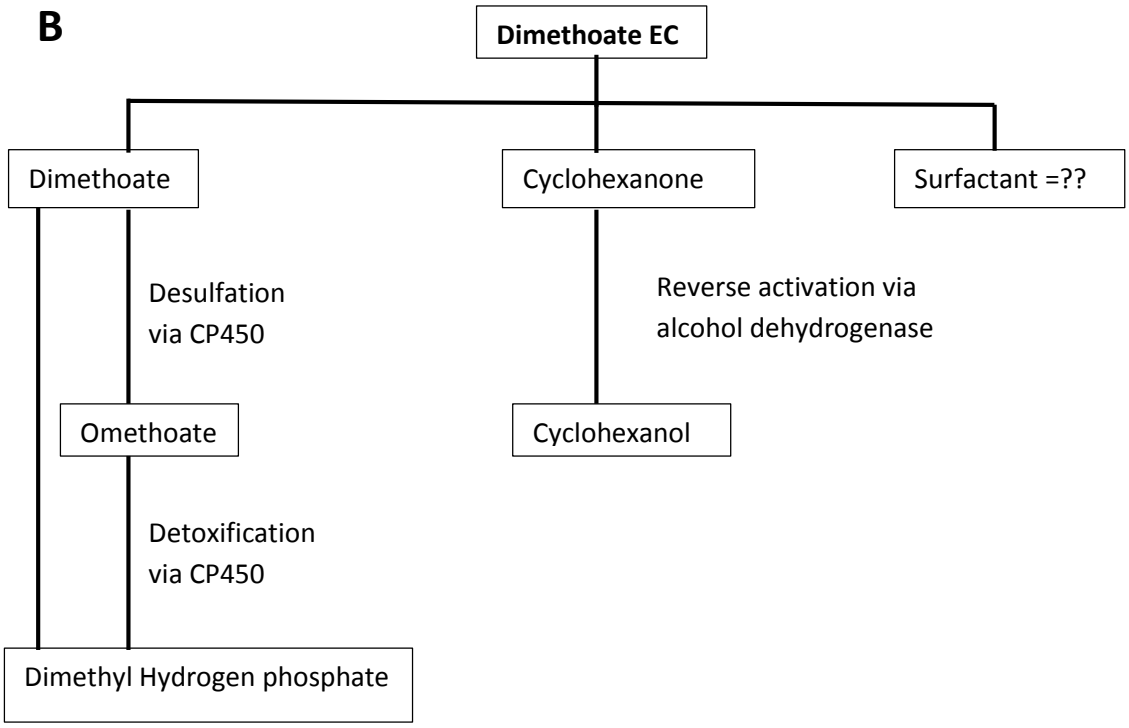
Solvents also undergo metabolism inside the body. For example, cyclohexanone undergoes reduction to cyclohexanol, a process mediated by reverse activation of alcohol dehydrogenase, which is strongly expressed in the liver and stomach lining in most humans. However, some studies indicate that apart from formation of cyclohexanol, there is a significant production of cyclohexanediol (Mraz et al., 1994). Xylene, another commonly used solvent in pesticide formulation, also undergoes metabolic transformation before excretion via urine. Xylene is mostly excreted as a conjugated form “toluric acid”. A lesser amount is excreted as xylenol (Sedivec and Flek, 1976; Miller and Edwards, 1999). During the course of metabolism, xylene also utilizes both alcohol dehydrogenase and aldehyde dehydrogenase enzyme similar to cyclohexanone metabolism (Fig 1.1). 4-Methylpyrazole (Fomepizole) is a competitive inhibitor of alcohol dehydrogenase, which is commonly used in ethanol and ethylene glycol intoxication in humans (Baud et al., 1986).

Fig1.1: Schematic diagram of pesticide metabolism. A: Three main components present in commercially available pesticide, B; metabolism of commercial pesticide Dimethoate EC inside human body.

A


Commercial Pesticide
Active ingredient / Anticholinesterase
Solvent
Surfactant

B


Dimethoate EC
Dimethoate
Cyclohexanone
Surfactant =??
Desulfation via CP450
Reverse activation via alcohol dehydrogenase
Omethoate
Cyclohexanol
Detoxification via CP450
Dimethyl Hydrogen phosphate

1.1.6 Animal models for studying pesticide toxicity

OP pesticide toxicity has been studied using several animal models, including rodents, primates and zebra fish (Yen et al., 2011). However, the confounding factor behind pesticide toxicity in most previous studies is lack of evidence on effects of commercial pesticides rather than their purified ingredients. Almost all previous studies have used only the presumed active ingredient (anticholinesterase) to determine the effects. There are only very few studies that have examined the effects of commercial product (Casida and Sanderson, 1961; Eddleston et al., 2012). It has been shown that the LD50 for pesticide is less than 50% of that of the active ingredient alone (Casida and Sanderson, 1961). The use of rodent animal models to understand pesticide toxicity is still a debatable question as they have greater capacity for metabolic detoxification of OPs compared to humans (Wrighton and Stevens, 1992). Due to similarities to human detoxification and excretion of drugs, use of minipigs in toxicology studies have become more important (Svendsen, 2006; Forster et al., 2010b; Forster et al., 2010a). Considering the relevance of the animal model for toxicological studies (minipigs show similar detoxification and excretion of drugs with humans) and the use of matterials (commercial pesticide which contains both active ingredient and solvent), Eddleston et al (2012) intensive minipig model of OP toxicity has an important bearing on understanding mechanisms of OP pesticide toxicity in human.

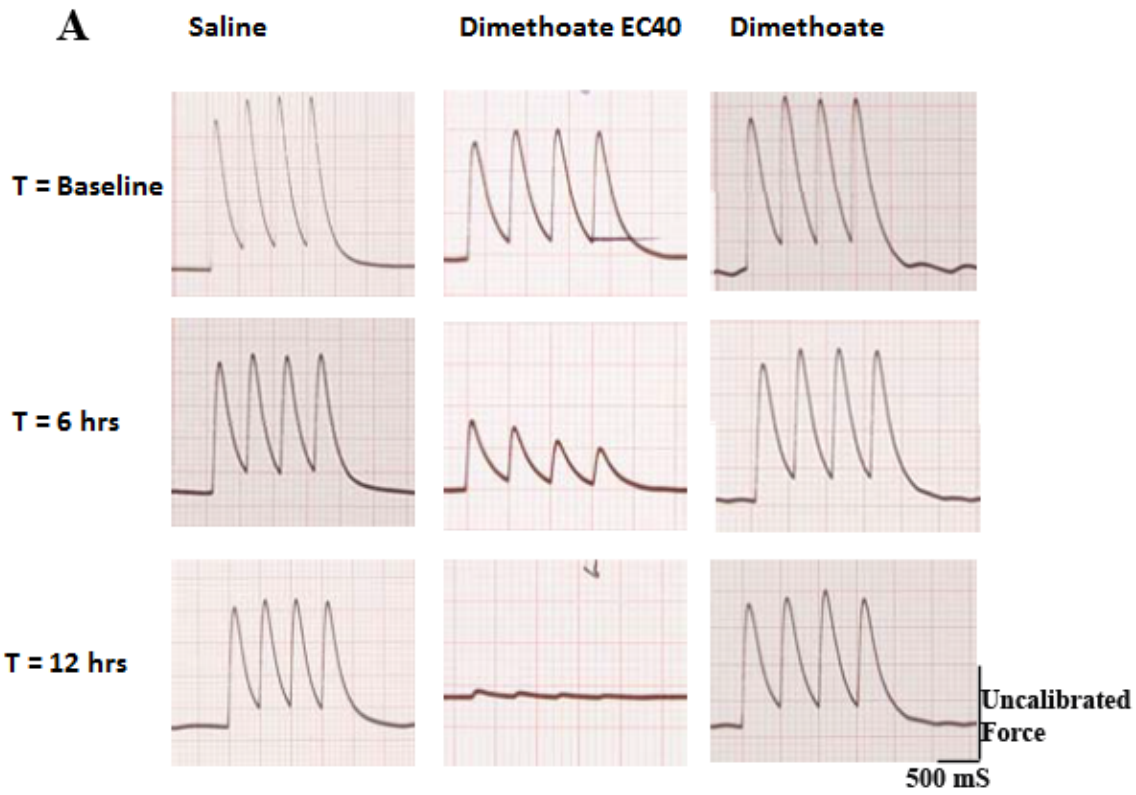
1.1.7 Effects of dimethoate EC40 ingestion in a minipigs model

Dimethoate EC belongs to the class of moderately toxic pesticides (WHO class II), while producing higher fatalities compared to other class II pesticides (20.6%) in humans (Dawson et al., 2010). Eddleston et al (2012) have shown that poisoning of minipigs with commercial pesticide dimethoate EC40 demonstrated a similar clinical picture to that of human patients, including severe cardiovascular shock, neuromuscular transmission dysfunction and insensitivity to pralidoxime (antidote for AChE inhibition) therapy. Most importantly, they have shown that severe toxicity arose from the combination of the OP active ingredient and solvent rather than the OP alone. Absence of solvent (dimethoate alone) did not produce NMJ dysfunction, along with substantial reduction in cardio-toxicity.

In Eddleston's model Göttingen minipigs were first anaesthetised and cardiorespiratory functions were maintained at an optimal level. The animals then received either formulated (commercial product) or unformulated (active ingredient only) pesticides, or saline control, via oral gavage. Minipigs were maintained for 12 to 48 hours, using different

pharmacological interventions as required: for example noradrenaline to overcome hypotensive shock. Mechanomyography was used to monitor *in-vivo* neuromuscular function. Trains-of-four (TOF) stimuli were applied at 2 Hz, at intervals greater than 10 s. The use of TOF to monitor neuromuscular blockade is common medical practice among surgeons (Fuchs-Buder et al., 2009). In the minipig model, complete loss of NMJ transmission was apparent about 12 hours from post treatment with pesticide dimethoate EC40 (commercial product). However, there was no indication of neuromuscular block either with dimethoate alone or saline control (Fig 1.2).

Fig. 1.2: Data from minipig model of OP toxicity A; Mechanomyography traces showing relative (uncaliberated) force from a minipig model of organophosphorus toxicity. Dimethoate EC40 treated minipigs showed apparent loss of neuromuscular function after 12 hours post treatment, while saline or dimethoate only treatments did not cause any functional loss. This observation suggests that both dimethoate (anticholinesterase) and cyclohexanone (solvent) is required to produce neuromuscular transmission block B: Plasma concentration levels of pesticide ingredients and their metabolic breakdown producs after oral treatment of Dimethoate EC40. Note that concentrations of dimethoate, omethoate, cyclohexanone and cyclohexanol stabilized after about 8hrs at 1mM, 100µM, 1mM and 5mM respectively. (Figure from Eddleston et al 2012).


A
Saline
Dimethoate EC40
Dimethoate
T = Baseline
T = 6 hrs
T = 12 hrs
Uncalibrated Force
500 mS
B
F
Plasma concentration (µM)
1200
900
600
300
200
100
0
Dimethoate
Omethoate
0 2 4 6 8 10 12
Hrs post-OP administration
G
Plasma concentration (mM)
6
5
4
3
2
1
0
Cyclohexanol
Cyclohexanone
0 2 4 6 8 10 12
Hrs post-OP administration

1.1.8 Metabolism of dimethoate and cyclohexanone

As stated above, dimethoate and cyclohexanone undergo metabolic conversion to omethoate and cyclohexanol, respectively (Fig. 1.1).

Dimethoate is converted into its oxygen analogue omethoate through a desulfuration pathway in the liver that utilises cytochrome P450 enzyme. Due to a higher level of accumulation in the body and increased appearance in urine it is thought that omethoate has more stability to enzymatic hydrolysis than dimethoate (Lucier and Menzer, 1970). Both dimethoate and omethoate formulate a hydroxyalkyl compound, which then undergoes further dealkylation by liberation of aldehyde group then excretion via urine (Lucier and Menzer, 1970).

Cyclohexanone undergoes metabolic reduction to form cyclohexanol via the alcohol dehydrogenase enzyme. Glucuronide-conjugated cyclohexanol is the major urinary excreted form in humans and other animals (Sakata et al., 1989; Mraz et al., 1994). However, the presence of 1,2-cyclohexanediol and 1,4- cyclohexanediol in urine after acute exposure to cyclohexanone has been identified in humans (Mraz et al., 1994).

1.1.9 Preliminary hypothesis

Based on the findings reported by Eddleston et al (2012), my preliminary hypothesis was that failure of synaptic transmission at NMJ with OP pesticide poisoning is not entirely due to anti-acetylcholinesterase activity but, rather, due to the combinatorial actions of both the anticholinesterase active ingredient and the solvent present in the pesticide.

I have examined my primary hypothesis in this thesis using commercial pesticide Dimethoate EC40, similar to Eddleston et al (2012). Before itemising the detailed objectives, I consider first the relevant physiological and pharmacological properties of mammalian NMJs.

1.2 Structure and function of the neuromuscular junction (NMJ)

NMJs are one of the most highly examined synaptic sites in vertebrates due to their apparent simplicity and accessibility. They had been studied using many techniques such as electrophysiology and physiological imaging (Del Castillo and Katz, 1954; Takeuchi and Takeuchi, 1959; Wood and Slater, 1997; Gillingwater et al., 2002; Ribchester et al., 2004; Melom et al., 2013), electron microscopy (Heuser and Reese, 1973; Desaki and Uehara,

1981; Nagwaney et al., 2009) and immunofluorescence labelling (Court et al., 2008) in order to understand synaptic formation, function and plasticity (Costanzo et al., 2000; Ribchester, 2009).

1.2.1 Muscle contraction and its regulation (motor unit, twitch, tetanus)

A motor unit consists of a motor neuron and the skeletal muscle fibres innervated by its axonal terminals. This is the basic functional unit of co-ordinatation of muscle contraction. The force of the muscle contraction can be increased by recruiting more motor units and by increasing the frequencies of the action potentials that drive them. Experimental paradigms yield two types of contraction: isometric (constant length) and isotonic (constant force or load) contractions (Berne, 1992).

When a single stimulus is applied to the nerve, a single action potential will be elicited in the muscle fibres it innervates and, after an activation delay, the muscle will contract. This single contraction is called a twitch contraction (Brown and Matthews, 1960)**.** The twitch tension rises to a maximum within 10 – 50 ms and then more slowly returns to resting tension, with a time course that depends upon the particular muscle. Fast twitch muscles has faster rise time, while slow twitch muscles take longer time to reach maximum twitch tension (Buller et al., 1960).

A second stimulus, applied before the muscle has completely relaxed, induces another contraction that adds to the first, the sum of the tensions becoming greater than that of a single twitch. This is called summation**.** When muscle is stimulated at a higher frequency, the tension rises to a more-or-less steady value, much greater than the twitch tension, and this is referred to as a tetanus, or tetanic contraction**.** When the individual twitch contributions to the tetanus can still be seen in the record, this tetanus is called unfused or incomplete. However, at higher stimulation frequency, the maximum tension the muscle can sustain is obtained and individual twitches are no longer discernible, and this is called a fused or complete tetanus. Slow twitch muscles produce fused tetanus at low frequencies (e.g. 15Hz) while fast twitch muscles produce fused tetanus at higher frequencies (e.g. 60Hz).

1.2.2 History of research on the NMJ: a model chemical synapse

As defined classically by C.S. Sherrington, the point of functional contact between a neuron and its target (another neuron, gland or muscle) is called a synapse. Following initial debate

over whether transfer of signals from neurons to their targets occurs mainly through either direct electrical interaction or via chemical interaction (neurotransmitter), it is now universally accepted that most synapses in the mammalian nervous system, including NMJ, are chemical synapses which involves release of neurotransmitter (ACh in the case of NMJs) from the presynaptic portion (motor nerve terminal).

The first idea of a chemical synapse was generated by Elliot in 1904, while Loewi in 1921 demonstrated the chemical nature of transmission in the autonomic nervous system via a simple experiment utilising the effect of perfusate collected from an isolated frog heart produced by stimulation of the vagus nerve (Nicholls, 2012). The ACh action in neuromuscular transmission was establishment by Dale and his colleagues (Dale et al., 1936). The use of NMJ as a model chemical synapse was pioneered by Fatt and Katz (1951) with their intracellular recording of MEPP at frog NMJs. This was then taken further to establish the quantal hypothesis, which is the modern day understanding of transmitter release from chemical synapses (Katz, 1996).

1.2.3 Structure and ultrastructure of NMJ

NMJs comprise three main compartments: presynaptic/motor nerve terminal, post synaptic/folded skeletal muscle surface, and synaptic cleft and four cell types: motor neurons, muscle fibres, terminal Schwan cells and kranocytes (Court et al., 2008) (fig. 1.3, 1.4).

Muscle fibres originate from mesodermal cells to produce myotubes, which then mature to produce peripherally nucleated muscle fibres. Motor neurons are derived from the ventricular germinal zone in the neural tube. Their axons progress through peripheral nervous to muscles where they branch and innervate muscle fibres (Douglas and Ribchester, 1986). Schwan cells have a neural crest cell origin and follows motor axons until they reach their final targets (Grinnell, 1995; Sanes and Lichtman, 1999). Initially, muscle fibre innervation is polyneuronal (muscles fibres are innervated by two or more motor inputs). The post natal maturation leads to removal of the convergence by the process referred to as synapse elimination, resulting in mononeuronal innervation of muscle fibres (Brown et al., 1976; Betz et al., 1979). This synapse elimination is influenced by competition - a process which utilises activity-dependent and -independent process (Ribchester and Barry, 1994; Costanzo et al., 2000; Walsh and Lichtman, 2003; Favero et al., 2012).

Fig 1.3: Schematic diagram of NMJ demonstrating different types of cells associated. Micrograph from Court et al (2008).

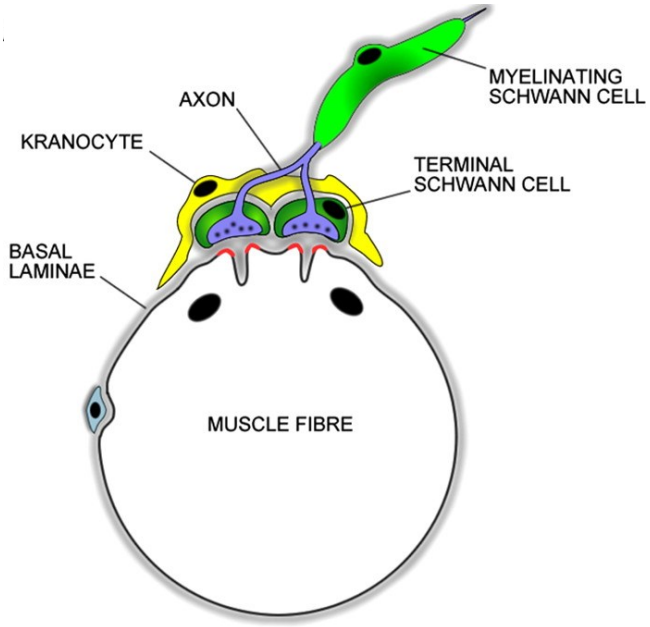
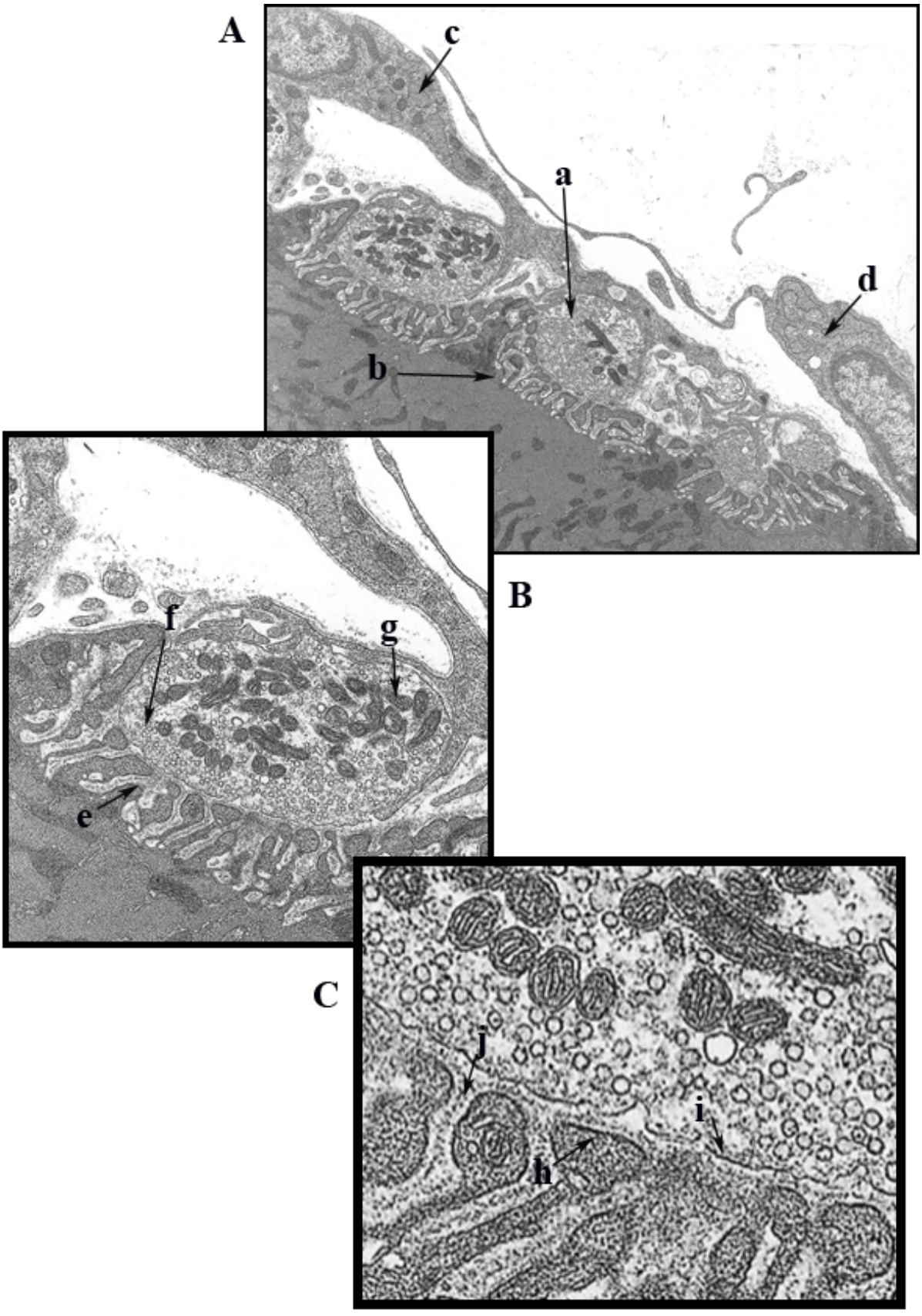

MYELINATING
SCHWANN CELL
AXON
KRANOCYTE
TERMINAL
SCHWANN CELL
BASAL
LAMINAE
MUSCLE FIBRE

Fig 1.4: Cellular architecture of NMJ. A: Electron micrograph showing four cell types of NMJ a)motor nerve terminal, b) motor endplate, c) Schwan cell, d) kranocyte. B: enlarged synaptic bouton, e) junctional folds, f) synaptic vesicles, g) mitochondria. C: enlarged NMJ, h) post synaptic densities, i) active zones, j) synaptic cleft. Electro micrographs from Court et al (2008).


A
c
a
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The primary function of the motor nerve terminal is neurotransmitter release. The ultra-structural hallmark of nerve terminals are the synaptic vesicles: 30nm spheres that contain the neurotransmitter acetylcholine (ACh), which cluster in the distal half of the terminal that faces the muscle fibre. Most of the mitochondria present in the motor nerve terminal are gathered in the proximal half of the terminal. The other important structural hallmark of nerve terminals are the active zones (dense patches on terminal membrane, associated with voltage gated K^+ and Ca^{2+} ion channels, and many proteins including syntaxin, SNAP 25), where synaptic vesicles bind and release their contents into the synaptic cleft (Ruiz et al., 2011; Sudhof, 2012, 2013). Other significant protiens in the nerve terminal include choline acetyltransferase (ACh synthetic enzyme) and a vesicular ACh transporter (which transport ACh in to the synaptic vesicles) and multiple presynaptic membrance channels and receptors (see below).

The main post synaptic features are proteins associated with detection of neurotransmitter ACh such as ACh receptors, ion channels and associated binding proteins.The skeletal muscle surface has many structures that optimize synaptic transmission, including a high density of ACh receptors in junctional folds (~ 1μm deep) (Sanes and Lichtman, 1999) (>10,000 / μm^2) (Salpeter & Loring, 1985) that open directly opposite active zones. In addition voltage-sensitive sodium ion channels are concentrated in the crypts of the junctional folds (Slater, 2009). The arrangement enhances efficacy of synaptic transmission (Wood and Slater, 1997). There is also presence of neural cell adhesion molecules concentrated along with the sodium ion channels (Covault and Sanes, 1986). The heterogeneous composition of the cytoskeleton of the junctional folds involves generating and maintaining the different domains within them (Sanes and Lichtman, 1999).

The synaptic cleft is the space (50nm) between the pre and post synaptic surface. It is infiltrated with extracellular matrix: the basal lamina. Acetylcholinesterase (AChE) enzyme is contained within the synaptic basal lamina and projected into junctional folds (McMahan et al., 1978). Along with the AChE, there are many other molecules present such as collagen IV, laminin, glycoconjugates and signalling molecules (agrin and neuregulin) (Sanes and Lichtman, 1999). The basal lamina determines and coordinates the pre-synaptic differentiation of the active zones and the post-synaptic accumulation of AChRs, as well as secondary folds facing the active zones (Massoulie and Millard, 2009).

1.2.4 Axonal action potential and presynaptic voltage-gated ion channels

Axonal action potential propagation to the NMJ is via "saltatory conduction". This is a mode of conduction which action potential leaps from one node of Ranvier (active region on the axon, containing voltage-gated sodium channels) to the next (Slater, 2009). Upon reaching the nerve terminal, the action potential influences many different types of ion channels in order to trigger the exocytosis of the synaptic vesicles.

There are many types of ion channels present in the presynaptic nerve terminal. Among these, voltage gated ion channels are important in process of neurotransmitter release, namely Ca^{2+}, K^+, Na^+, Cl^-, and non-selective ion channels. Among the various sub types of Ca^{2+} channels present (L, N, NTP, N_t, P, P and /or Q and Q) (Meir et al., 1999) mammalian motor nerve terminal contain "N" type voltage gated Ca^{2+} channels (Hamilton and Smith, 1992) and P/Q type (Plomp et al., 1992; Uchitel et al., 1992). Mammalian motor nerve terminal also contain three sub-types of K^+ channels; namely delayed rectifier K^+ channels, Ca^{2+}-gated K^+ channels and ATP-sensitive K^+ channels (Tabti et al., 1989).

1.2.5 Neuromuscular transmission: EPP/EPC; quantal analysis; and non-linear summation (NLS)

Transmitter release from nerve terminal occurs in following series of steps (Meir et al., 1999).

The propagation of an action potential into the nerve terminal is the first step of evoked transmission. This will lead to presynaptic activation of different molecules causing the depolarization of the presynaptic membrane. This membrane depolarization influences large numbers of voltage-gated ion channels such as Na^+, Ca^{2+}, K^+, Cl^- and some nonselective channels. The opening of Ca^{2+} channels allow entry of Ca^{2+} ions into the nerve terminal, which then binds to intracellular Ca^{2+} sensors (e.g. synaptotagmin) (Bommert et al., 1993). Ca sensor activation leads to activation of vesicular and terminal SNARE protieins in the nerve terminal. This activation of fusion machinery leads to fusion of synaptic vesicles with the surface membrane at active zones and induces rapid release by exocytosis of multi-molecular "quanta" of ACh, from synaptic vesicles into the synaptic cleft (Katz and Miledi, 1969). This fusion machinery involved is mediated by SNARE proteins, which includes synaptobrevin, syntaxin and SNAP-25 (Sudhof, 2012, 2013).

Spontaneous release of vesicles occurs normally at low frequency (1/sec) producing small (1mV) depolarization at postsynaptic membrane (MEPPs) (Fatt and Katz, 1951; Liley, 1956).There is also a low leakage of ACh from nerve terminal, referred to as non-quantal release, but this does not normally cause a substantial endplate depolarization either at rest or following nerve excitation (Vyskocil et al., 2009).

The ACh molecules released from the nerve terminal diffuse through the synaptic cleft and then bind to ligand-gated ACh receptor ion channels. This will initiate conformational change in the AChR and opening of the ion channel, leading to changes in the ion permeability either side of membrane, thus initiating a net inward ionic current (endplate current, EPC) due to influx of Na^+ ions into the post synaptic cell and K^+ ionic efflux into the synaptic cleft. The resulting depolarization, the EPP, causes opening of voltage-gated sodium ion channels, which then initiate a muscle action potential, and finally contraction of the muscle cell via excitation-contraction coupling (Slater, 2009). The EPCs have a rapid rise and slower exponential decay which is determined by the rates at which the AChR switches among resting, intermediate and open states (Magleby and Stevens, 1972). EPPs have longer time course as the endplate current must discharge through the passive membrane resistance and capacitance of the muscle fibre. Most of the ACh released from the presynaptic terminal is hydrolysed to acetate and choline by the action of AChE either before reaching the post-synaptic membrane or after interacting with receptors, thereby terminating synaptic transmission. Choline is taken up in to motor nerve terminal by choline-transporter and utilized in the re-synthesis of ACh. Synaptic vesicles are recycled by endocytosis and the recycled vesicles refilled with ACh for subsequent re-utilization (Betz and Wu, 1995; Betz and Angleson, 1998).

The idea that multi-molecular “quanta” of transmitter were released from presynaptic terminals was first suggested by B. Katz and his colleagues in middle of the 20th century (Fatt and Katz, 1951; Del Castillo and Katz, 1954, 1956; Katz and Miledi, 1969). They established a statistical model, which could describe the fluctuation of “quantal content” of the EPP in different situations by relating it to the amplitude of the unitary quantal component: that is, miniature EPPs (MEPPS) (Fatt and Katz, 1951; Del Castillo and Katz, 1954, 1956; Katz and Miledi, 1969). According to this model, the quantal content of an EPP can be estimated by dividing the amplitude of the EPP by the “quantal size”, equivalent to the mean amplitude of MEPPs. This is called the Direct Method for estimating quantal content.

$$m = \frac{mean\ EPP\ (or\ EPC) amplitude}{mean\ MEPP\ (or\ MEPC) amplitude}$$

The Direct Method suffers from the effects of non-linear summation (see below) but accurate estimates of quantal content may be obtained from EPCs and MEPCs instead, since non-linear summation does not occur when ionic currents are measured at a fixed membrane potential, for instance under voltage clamp.

According to the "quantal hypothesis", the mean quantal content (m) is a product of the number of quanta / vesicles available for release (*n*) and the probability of their release (*p*).

$$m = n.p$$

Further consideration of this binomial relationship, leads to the general prediction that probability of release of x quanta (P(x)) is given by:

$$P(x) = \frac{n!}{(n-x)!\,x!} \,.p^x.q^{(n-x)}$$

The quantities n and p cannot easily be determined independently. However, simpiifying assumptions can be made that the number of quanta released by any given stimulus is very much less than the number available (x<<n) and the probability of release of each vesicle is very much less than unity (p<<1). Under these mathematical conditions, the relationship between P(x) and m simplifies to a Poisson distribution:

$$P(x) = \frac{m^x}{x!} \,.\exp(-m)$$

In normal physiological synaptic transmission, evoked EPPs produced by nerve stimulation reach the threshold level and trigger a muscle action potential. However, transmission can be depressed by blocking ACh receptors (using a nicotinic receptor antagonist such as d-tubocurarine) or by reducing the probability of quantal release through increasing the Mg^{2+} ion concentration and reducing the Ca^{2+} ion concentration in the medium (Del Castillo and Katz, 1954; Dodge and Rahamimoff, 1967).

Two alternative methods for estimating quantal content arise from the analysis that led to the Poisson equation. These methods are commonly referred to as the Method of Failures and the Variance Method.

When the mean quantal content is low, some of the nerve stimulation does not produce an evoked response. This low quantal content is achieved in experimental conditions by reducing the Ca^{2+} and or increasing the Mg^{2+} ion concentration in the bathing medium (Dodge and Rahamimoff, 1967). This bathing medium leads to reduction in probability of release (p), thereby reduced mean quantal content. Under these conditions, quantal content can be estimated using the "Failures method". From the Poisson distribution:

$$P\ (0) = \exp(-m)$$

The probability of failures, P (0), is determined experimentally by counting the number of failures divided by the number of stimuli. Taking natural logarithms and rearranging the above equation gives:

$$m = Ln\ \frac{number\ of\ stimuli}{number\ of\ failures}$$

Another property of the Poisson distribution is that its variance equal its mean (Hubbard et al., 1969).

It can be shown that: $Variance\ \left(\frac{v_1}{q}\right) = V/q$

Where v_1 is individual EPPs amplitude, q is mean MEPP size and V is mean EPPs amplitude. It can be shown that

$$q = variance\ (v_1)/V$$

Thus, an alternative method of estimating quantal content is the "Variance method", which employs the mean and variance of EPP amplitudes:

$$m = \frac{(mean\ EPP\ amplitude)^2}{(variance\ of\ EPP\ amplitude)}$$

This is also referred in terms of the coefficient of variation (C.V= standard deviation / mean):

$$m = C.V.^{-2}$$

The reliance on EPP in estimating quantal content can produce errors in estimated *m* due to non-linear summation of the EPP. This is particular a problem when the EPPs are large, e.g.

higher m. Non-linear summation arises because as the amount of transmitter released onto the post synaptic membrane increases, the depolarization produced by each quantum decreases. In another words, as the driving force decreases (difference between the resting membrane potential and the reversal potential for the action of ACh at its receptor), the EPP amplitude reduces. The Direct method of quantal analysis is based on EPP: MEPP ratios, can therefore lead to underestimates of m, while Variance method can produces overestimates (Martin, 1955; Wilson, 1977). The non-linear summation of the EPP amplitude can be corrected mathematically using following equation (Martin, 1955; McLachlan and Martin, 1981).

$$V' = V / ((1 - f.V / E)$$

Where V' is the corrected EPP amplitude, V is the observed EPP amplitude. E is the driving force (difference between the resting membrane potential and the reversal potential). The correction factor f is an arbitrary value which varies from the muscle fibre to muscle fibre depending on its length, diameter and membrane resistance (McLachlan and Martin, 1981).

Use of EPC (and MEPC) to estimate quantal content is a more reliable method as EPC amplitudes do not need to be corrected for non-linear summation since the driving force remains constant at a voltage-clamped neuromuscular junction. EPCs therefore sum linearly at a constant membrane potential (McLachlan and Martin, 1981; Wood and Slater, 2001).

1.2.6 Mechanisms of exocytosis and vesicle recycling:

Exocytosis of the synaptic vesicles is associated with interaction of two set of proteins present in synaptic vesicles (V-SNARES – synaptotagmin and synaptobrevin) and nerve terminal membranes (T-SNARE – syntaxin and SNAP-25). Following "docking" of the synaptic vesicles at the active zones, vesicles are then turn in to "primed" state assisted by ATP-dependent step, before release from the terminal (Slater, 2009). Further increase in cytoplasmic Ca^{2+} level converts "primed" vesicles in to full fusion (Stanley, 1997). This triggers the conformational changes in the synaptic terminal and causes fusion, thereby releasing transmitter into the synaptic cleft (Slater, 2009; Sudhof, 2012, 2013).

Following exocytosis, recovery of the nerve terminal membrane normally occurs through endocytosis. Recycled vesicles are then taken in to the nerve terminal and transferred to vesicle "pools" (recycling pool and reserve pool) (Slater, 2009; Ruiz et al., 2011)). ACh is

then transported in to the recycled vesicles by the vesicular cholinergic transporter (VAChT) thereby preparing for the subsequent transmitter release (Erickson and Varoqui, 2000).

1.2.7 Drugs affecting transmitter release, action and breakdown

Synthesis, storage, release, action and inactivation of the neurotransmitters are the major target mechanisms affected by drugs.

Ethylcholine mustard aziridinium ion inhibits choline acetyltransferase (ChAT)) while hemicholinium-3 blocks the membrane transporter responsible for uptake of choline. These compounds therefore inhibit the synthesis of neurotransmitter ACh (Rang, 2003).

Filling of the synaptic vesicles by ACh is prevented via inhibition of the ACh membrane transporter by vesamicol (Parsons et al., 1993).

Drugs affecting release mechanisms mainly act as either inhibitors or enhancers. A high concentration of Mg^{2+} ions competes with Ca^{2+} ions and depresses exocytosis, while ω – agatoxin inhibits the voltage gated Ca^{2+} ion channels ("P/Q" sub type), thereby blocking transmission. Botulinum toxins block transmitter release by binding to and enzymically cleaving the SNARE proteins involved in transmitter release (Simpson, 2004). Drugs that enhance transmitter release include 4–aminopyridine, which blocks presynaptic potassium channels, and α-latrotoxin which tonically permeabilises presynaptic membranes to Ca^{2+} and causes substantial asynchronous release and depletion of synaptic vesicles from nerve terminals (Henkel and Sankaranarayanan, 1999).

Transmitter action is also affected by drugs by that act either as inhibitors (antagonists) or enhancers (agonists). Antagonists bind to ACh receptors and thereby prevent formation of ACh-receptor complexes. Nicotinic antagonists include d-tubocurarine and α- bungarotoxin. There is another set of anatgonists that act on ACh receptors by inducing allosteric changes in ACh receptors, or steric hindrance to ACh binding, thereby reducing the affinity of receptors for ACh molecules. These include phencyclidine and methyl violet-10B. Drugs that act as agonists of ACh neurotransmitter action include carbachol, decamethonium (which prolongs the open state of the ligand-gated channels) and suxamethonium, which act as a depolarizing blocker through initial sustained activation of ACh receptors, then maintained depolarization leading to inactivation of voltage-gated Na-channels.

The group of drugs and compounds acting on inactivation of the neurotransmitter by AChE include carbomates (e.g. neostigmine, pyridostigmine, physostigmine) and OPs (e.g. echothiopate, sarin, malathion, dimethoate,). These drugs and compounds act as reversible or irreversible inhibitors of acetylcholinesterase molecule.

1.2.8 Determinants of safety factor for neuromuscular transmission

The high safety factor of the neuromuscular transmission is also one of the important elements of reliable transmission at NMJ. Safety factor is referred to as "ability of neuromuscular transmission to remain effective under various physiological conditions and stresses" (Wood and Slater, 2001). The safety factor at NMJ is a consequence of the amount of transmitter released per nerve impulse exceeding that required to trigger an action potential in the muscle fibre by a factor of 2 – 5 (Wood and Slater, 2001). There are many elements that contribute to safety factor including quantal release, AChE activity, ACh receptor numbers, density of post synaptic Na^+ channels, and architecture of postsynaptic folds (Wood and Slater, 2001).

The reliability or effectiveness of neuromuscular transmission depends on the size and molecular organization of the NMJ and how the molecules that mediate transmission are distributed in it (Slater, 2009). These ensure the reliability of NMJ transmission, without failures, even after intense voluntary exertion (Bigland-Ritchie et al., 1978). Reduction in reliability cause severe impairment in overall neuromuscular transmission. Inherited or acquired reduction in reliability of transmission leads to fatigue, weakness and paralysis of the muscle (Slater, 2009).

1.2.9 Passive electrical properties and action potentials in muscle fibres

Passive membrane properties of the muscle affect several features: a) the size and magnitude of the endplate potential, b) the time course of the membrane potential after current injection, c) the distance that depolarization will travel, and d) the speed of the potential propagation. These membrane properties include membrane resistance (R_m), membrane capacitance (C_m) and intracellular axial resistance (R_a). The membrane and axial resistances can be lumped together in a quantity referred to as the "input resistance" (R_{in}), which is principally determined by the diameter or cross-sectional area of the muscle fibre and membrane permeability at a given membrane potential. Empirically (ie experimentally), R_{in} is determined by measuring the steady-state voltage response following injection of a small

constant step current ($R_{in}=\Delta V/\Delta I$). The property of membrane capacitance also confers a delay in the voltage response to a step current. The time delay is indicated by the membrane time constant (τ).

The commonly used experimental technique determining these passive properties include applying a step current pulse through a membrane (first electrode) and to measure the time required for the membrane potential to reach a steady level using a second electrode inserted in to the same fibre with minimum distance (typically less than 100 μm) from the first one.

Input resistance determines the EPP amplitude, and this depends on the density of the open channels at rest (how leaky the muscle membrane is) (R_m), axial resistance (R_a) and diameter of the muscle fibre (d). Input resistance is related to R_m and R_a by the following equation (Jack et al., 1983):

$$R_{in} = \frac{1}{\pi}\sqrt{[\left(\frac{R_m R_a}{d^3}\right)]}$$

Therefore, smaller diameter and less leaky muscle fibres will have larger input resistance.

The lipid bi-layer of the cell membrane also acts as capacitance. Membrane capacitance (C_m) also affects the time course the EPP. Membrane capacitance depends on the specific capacitance (C_m) and muscle fibre diameter. Cin is also defined by the following equation.

$$C_{in} = C_m(4\pi d^2)$$

Cm is a constant due to the uniform thickness of the membrane; therefore larger fibres will have higher membrane capacitance. Current flow will first charge the capacitance and then flow through the resistance. Therefore, as the capacitance charge, the amount of current flowing through the resistance increases until it reaches a steady stage voltage. The rate of change in this voltage is called membrane time constant (τ).

$$\tau = C_m R_m$$

The larger the τ is, the longer it takes to reach max voltage and slower the decay from the maximum voltage.

For the approximation of spherical cell, the time course of the response to a step current injection is given by:

$$\Delta V_m(t) = I_m R_{in} (1 - e^{-\frac{t}{\tau}})$$

Where I_m is membrane current. In this model, membrane time constant is determined by the time taken for the voltage to reach (1/e) or 63% of the steady-state voltage. However, the relationship between voltage and time constant is different in non-spherical cells.

The voltage and current relationship across the membrane in a "cable model" of the muscle fibre can be determined by combining Ohms law for the resistance with the time dependence on membrane capacitances.

$$\Delta V_m(t) = \left(\frac{R_a I_m \lambda}{2}\right) \text{erf}\left\{\left(\frac{t}{\tau_m}\right)^{\frac{1}{2}}\right\}$$

Where erf (x) $= \frac{2}{\sqrt{\pi}} \int_0^{\infty} \exp(-y^2) dy$. Which approximately grouping to a Gaussian curve (Equation 13.73 and 13.74 in Jack et al (1983)).

Since erf(1) = 0.84, V rises to 84% of its steady state value in one time constant (Jack et al., 1983). Therefore, time constant is measured from the 84% time to maximum for long cables (like TS muscle fibres) but about 63% for short cables (like FDB muscle fibres).

Axial resistance determines how far an impulse will travel. Current flow through the muscle fibre will face the axial resistance. The length constant, λ, describes the change in Vm at distance X, and this voltage change can be modelled using following equation.

$$\Delta V_m(X) = Voe^{-x/\lambda}$$

The length constant, λ, can be determined by using following equation.

$$\lambda = \sqrt{R_m/R_a}$$

Therefore, λ depends on the specific membrane resistance and axial resistance and membrane voltage will drop more steeply with distance the larger the diameter of the muscle fibre.

Similar to neurons, generation of action potential in muscle also requires depolarization of the membrane potential to the threshold level. Sub-threshold currents produced by the binding of ACh molecules to the nAChR trigger the opening of voltage gated Na^+ ion

channels (Na_v 1.4, sub type present in the skeletal muscle), which accumulate in the crypts of the postsynaptic folds. This activation causes regenerative entry of positive charge into the cells. This depolarization reaches the threshold level, leading to firing of an action potential in the muscle. During this time, positive charge entering at the NMJ is greater than that leaving in the surrounding region (Slater, 2009). Therefore, the excess charge depolarizes the adjacent membrane to threshold, setting in train the regenerative process that underlies the action potential.

In experimental conditions, Na_v 1.4 channels can be blocked using toxins such as conotoxin (μ-CTX) in order to abolish the muscle action potential, thereby facilitating the examination of EPP and underlying EPC (Braga et al., 1992; McIntosh and Jones, 2001; Ribchester et al., 2004).

1.2.10 Molecular mechanisms of excitation-contraction coupling and myofilament force generation

The process that links the action potential to cross-bridge cycling and contraction is called excitation-contraction coupling. This involves mainly three mechanisms; a) neuromuscular transmission and depolarization of the endplate membrane, sarcolemma, and t-tubules, b) the mobilization of Ca^{2+}, c) the action of Ca^{2+} on myofibrillar regulatory mechanisms that control cross-bridge cycling (Huxley, 1974).

Once an action potential is fired, the resulting surface depolarisation of the skeletal muscle is then carried into the cell via t-tubules (invagination of surface membrane). These t-tubules are connected to the sarcoplasmic reticulum (SR) (a specialized form of endoplasmic reticulum) at sites called “triads”. At triads, dihydropyridine receptors (voltage-dependent Ca^{2+} channels in t-tubules) interact with ryanodine receptors (Ca^{2+} release protein in the SR), thereby triggering Ca^{2+} release in to the cytoplasm, thus leading to a muscle contraction.

Muscle contraction is a result of an interaction between myofibrils. These myofibrils are organized to form array of distinct thick (myosin) and thin (actin, troponin**,** and tropomyosin) filaments. These filament arrays overlap to form sarcomeres. The heads of the myosin molecules project out from the thick filaments towards the thin filaments and these projections form cross-bridges / cross-projections**.**

The level of free Ca^{2+} in the cytoplasm regulates the muscle contraction. Maintenance of the free Ca^{2+} at a low level occurs via the sarcoplasmic reticulum, which contains energy dependent Ca^{2+} transporters.

Relaxation occurs when Ca^{2+} is pumped back into the sarcoplasmic reticulum. Subsequently, crossbridges are detached from the thin filaments and the troponin-tropomyosin regulated inhibition of actin and myosin interaction is restored. Finally, active tension disappears and the rest length is restored. This completes the contraction-relaxation cycle.

1.3 Acetylcholine (ACh), ACh Receptors (AChR) and ACh esterases (AChE)

Acetylcholine is an ester of acetic acid and choline which is synthesised in the presynaptic nerve terminal. The acetyl group from the acetyl-coenzyme A is transferred to choline by the enzyme choline acetyltransferase (ChAT) (Nachmansohn and Machado, 1943; Byrne and Roberts, 2004).

1.3.1 Molecular characterization of the postsynaptic AChR: activation and desensitization

The endplate AChRs are heteropentamers, comprising α, β, and δ subunits, along with either the fetal γ subunit or the adult ε subunit (Takai et al., 1985).

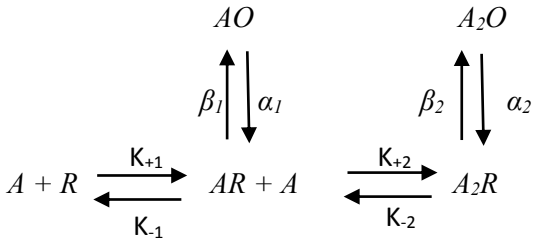
The mechanism of ACh binding to AChRs and forming agonist-receptor complex was first described by del Castillo & Katz (1957). However, the mechanism proposed, one agonist binding site per receptor, was quickly modified with evidence showing more than one binding site (Katz and Thesleff, 1957b).

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} AR + A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} A_2R \underset{\alpha_2}{\overset{\beta_2}{\rightleftarrows}} A_2O$$

In the case of identical binding sites, where A is the agonist, R is the resting receptor with the channel closed, AR and A_2R are the inactive complexes with the channel closed and A_2O is the active complex with the channel open. K_{+1} and K_{+2} are the agonist association rate constants, K_{-1} and K_{-2} are the agonist dissociation rate constants, β_2 is the channel opening rate constant and α_2 is the channel closing rate constant.

The desensitisation of motor endplate (Katz and Thesleff, 1957b) is a process that inactivates the majority of the receptors when the agonist concentration is maintained for a sufficiently

long time. First, it was described that to a large extent desensitization develops following steady-state exposure to agonist, suggesting that in the desensitized state an AChR binds agonist more tightly than in its resting state. Later it was shown that agonist affinity increases in a time dependent manner and onset of the affinity increase occurs in parallel to the onset of functional desensitization (Weiland et al., 1976; Weiland et al., 1977; Sine and Taylor, 1980). Manod et al (1965) constructed a model (the MWC model) that incorporated the concept of interconvered active and inactive stats of the receptors with different affinity for their agonist (ACh in the cleft). The MWC model was subsequently modified on the basis of single channel analysis and can be represented as follows.



Where A is the agonist, R is the resting state, O is the open channel state. K_{+1} and K_{+2} are the agonist association rate constants, K_{-1} and K_{-2} are the agonist dissociation rate constants, β_1 and β_2 are the channel opening rate constants and α_1 and α_2 are the channel closing rate constants.

More complex models have been proposed and these (see Sine, 2012) and the modified MWC model above is still widly regarded as the foundation for receptor desensitization and its kinetics.

1.3.2 Molecular pharmacology of nicotinic AChR: agonist, antagonists

Agonists are the molecules that bind to receptors and initiating biological response while antagonists bind to receptors and block the agonist mediated response (Rang, 2003)

Some of the agonists mediating biological response at nAChRs include ACh, nicotine, epibatidine, cytisine and anatoxin A (Meir et al., 1999).

d-Tubocurarine is one of the important prototypical competitive antagonists of the nAChRs. It is a non-depolarizing, competitive antagonist (Jenkinson, 1960). α-Neurotoxins (including α- bungarotoxin), which are from elapid snake venoms, also competitively antagonize the

nAChRs. However, they bind to nAChRs with exceptionally high affinity and therefore effectively irreversibly. Molecular mechanisms include multiple points of attachment, conformational changes at the binding site, conformational changes in the toxin, or a combination of all (Meir et al., 1999).

1.3.3 Presynaptic cholinergic receptors

The presence of neuronal nicotinic AChRs in peripheral nervous system has been identified in many species including mammals (Wessler et al., 1992). Similar to post synaptic nAChRs neuronal nicotinic AChRs also comprise five subunits, mainly α and β (Cooper et al., 1991). Unlike post-synaptic nAChRs, neuronal nAChRs are modulators of neurotransmitters (Bowman et al., 1990; Meir et al., 1999). It is appear to be that these receptors likely to be involved in the positive-feedback regulation of ACh release, hence acting as autoreceptors (Meir et al., 1999). There is also some evidence on avaialblility of neuronal muscarinic AChRs at NMJ (Caulfield, 1993; Santafe et al., 2003), which appear to have similar role as neuronal nicotinic AChRs, indicating possible modulating of ACh release (Slutsky et al., 1999; Santafe et al., 2003).

1.3.4 Localisation and biochemistry of AChE

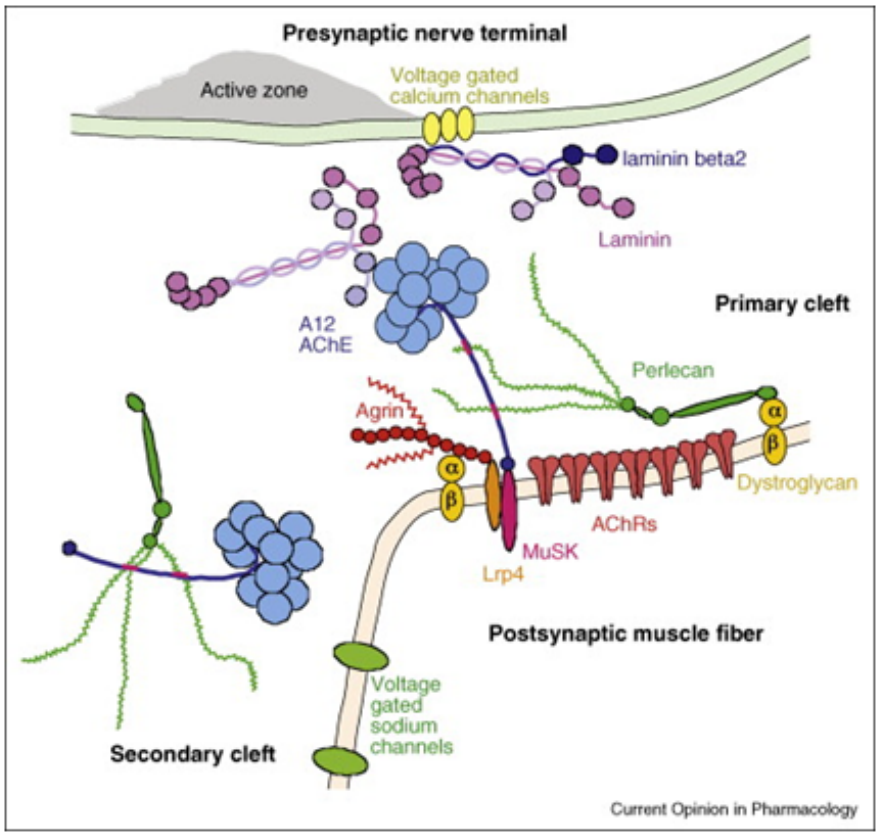
AChE is one of the fastest enzymes known (Nair et al., 1994): one AChE molecule degrades about 25,000 ACh molecules per second, (Quinn, 1987; Taylor and Radic, 1994). The enzyme is composed of homotetramers of globular catalytic subunits attached to a collagen tail (ColQ-AChE) (Massoulie and Millard, 2009) (Fig 1.5). Vertebrate AChE has many molecular forms, including $AChE_H$ (hydrophobic form) and $AChE_T$ (Tailed form), in which $AChE_T$ is present in adult muscle and brain (Massoulie, 2002). The deposition of the ColQ-AChE in the basal lamina depends on nerve-derived components, as well as muscle activity (Massoulie and Millard, 2009). The active site of the AChE molecule consists of 2 subunits, a peripheral anionic site and an esteratic sub site (Taylor and Radic, 1994); the esteratic sub-site is responsible for the hydrolysis of the ACh molecules. The anionic site interacts with the ACh quaternary ammonium atom and is responsible for its correct orientation (Taylor and Radic, 1994; Pohanka, 2011). This active site consists of a catalytic triad which include serine, histidine and glutamate protein molecules. The active site lies close to the bottom of a deep and narrow "gorge" (Sussman et al., 1991; Sussman et al., 1993; Taylor and Radic, 1994). The globular structure is lined with 14 hydrophobic residues (Sussman et al., 1991). The hydrolysis of ACh produces acetyl-enzyme and free choline. Then acetyl-enzyme

complex undergo acylation followed by de-acylation reactions in order to liberate acetic acid and regenerate the free enzyme (Taylor and Radic, 1994).

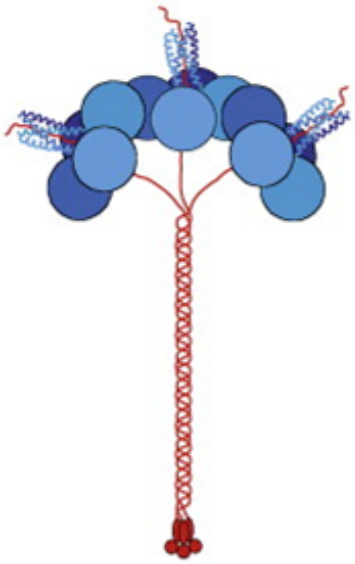
Mutations which produce an absence of the collagen tail result in muscle weakness (Massoulie and Millard, 2009). The anionic site of AChE enzyme also plays an important role in the development of Alzheimer's disease at CNS, as amyloid β peptide interacts with the peripheral anionic site. This may contribute to the formation of amyloid plaques and consequent damage to cholinergic neurons (Inestrosa et al., 2008; Pohanka, 2011).

Fig 1.5: Schematic diagram illustrating content of the synaptic cleft. A: Interactions among basal lamina, presynaptic and post synaptic molecules, B; Scheme of AChE molecules (catalytic domain subunits are in blue colour and collagen tail subunits are in red colour). (Diagram was adopted from Massoulie and Millard, 2009).

A


Presynaptic nerve terminal
Active zone
Voltage gated calcium channels
laminin beta2
Laminin
Primary cleft
A12 AChE
Perlecan
Agrin
α
β
Dystroglycan
AChRs
MuSK
Lrp4
Postsynaptic muscle fiber
Voltage gated sodium channels
Secondary cleft
Current Opinion in Pharmacology

B



1.3.5 History and mechanism of action of classic anticholinesterases

The use of anticholinesterase by humans can be dated back to centuries. The people of old Calabar (a city of south-eastern Nigeria) used to administer a Calabar bean mixture as an ordeal to persons accused of witchcraft or other crimes, as a punishment of their guilt. Many years later, it was identified that calabar beans contain physostigmine, a reversible cholinesterase inhibitor alkaloid (Fraser, 1867).

The inhibition of AChE by non-OP anticholinesterases (carbamates) occurs similar to OP induced AChE inhibition. In the presence of carbamates, AChE enzyme undergoes carbamylation, thereby inhibiting the activation of AChE (Wilson et al., 1960). However, carbamates bound to AChE hydrolyse spontaneously, thus reactivating the enzyme.

1.3.6 Therapeutic uses of anti-AChE

Therapeutic use of anticholinesterase can be traced to the 1870s' as physostigmine was used to treat glaucoma. Synthetic cholinesterase inhibitors were started to use as a therapy for skeletal muscle and autonomic disorders in 1930s'. Both pyridostigmine and physostigmine have been used for many years for the treatment of myasthenia gravis. Although the short-duration anticholinesterases are generally safe, reports of their misuse are associated with a clinical syndrome similar to pesticide intoxication. The first cholinesterase inhibitor to be tried for Alzheimer disease was tacrine, which was released for clinical use in 1993. However, it is no longer in clinical practice due to its adverse effects. Anticholinesterases drugs such as rivastigmine are now widely used for treating dementia in humans. Similarly, anticholinesterases metrifonate has been used to treat schistosomiasis and is undergoing trials for the treatment of primary degenerative dementia.

1.4 Organophosphorus toxicity

Organophosphate compounds are a diverse group of chemicals used in both domestic and industrial settings, which include insecticides (malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, ethion), nerve gases (soman, sarin, tabun, VX), ophthalmic agents (echothiophate, isoflurophate), and antihelmintics (trichlorfon). Herbicides (tribufos [DEF], merphos) are tricresyl phosphate–containing industrial chemicals.

1.4.1 History of OP: from nerve agents to pesticide

The first synthetic OP was made in 1854 by Philip de Clermount, who described the synthesis of tetraethyl pyrophosphate at a meeting of the French Academy of Sciences (Bajgar, 2004). Almost a century later, Lange and Schrader (1930) investigated the use of organophosphates as insecticides. Six years later, the first of the nerve agent intended for military use, known as "tabun" was synthesised by Schrader and he then synthesised second nerve agent "sarin" two years later (Sidell and Borak, 1992). Parathion was one of the first OP pesticide commercialized, which was invented by the Schrader in 1940s' following his discovery of nerve agents.

Massive organophosphate intoxication arose from suicidal, chemical warfare and accidental events. For example, the Jamaican gingerpalsy incident in 1930 affecting 30,000 – 50,000 people and accidental ingestion of ethion-contaminated food in a social ceremony in Magrawa, India in 2005, leading to 15 victims of OP toxicity. More importantly, chemical weapons (nerve gases) still pose a very real concern in this age of terrorist activity, including notable events such as in the battle fields of Iraq (1980s), sarin poison on a Tokyo subway (1995) and recent incident of sarin attack on the Ghouta agricultural belt around Damascus, Syria on the morning of 21 August (2014). However, the most substantial number of deaths occurs through the pesticide self-poisoning across the rural Asia (see Section 1.1) (Gunnell et al., 2007).

1.4.2 Mechanisms of inhibition of AChE by OP; antidotes and their mechanism

In the presence of the active OP compound, a serine hydroxyl group of the AChE molecule (the active site of AChE responsible for catalysis of ACh) is phosphorylated, inactivating the enzyme (Eddleston 2010). The inactive enzyme then undergoes one of two subsequent changes in state namely a) reactivation, either spontaneously or in response to drugs (oximes – see below) or b) "aging", which is loss of one alkyl group from the inactive enzyme, rendering the molecule permanently inactive (Fig 1.5). The rate of spontaneous reactivation of phosphorylated AChE enzyme depends on the chemical structure of OP compounds (Moretto, 1998; Vale, 1998). Ester groups attached to the phosphorus atom in OP compounds are mainly either two methyl (dichlorvos, dimethoate, malathion) or two ethyl groups (chlopyrifos, parathion). Dimethyl phosphorylated AChE enzymes have a rapid spontaneous reactivation; thereby patients should improve even without employing oxime therapy (Vale, 1998).

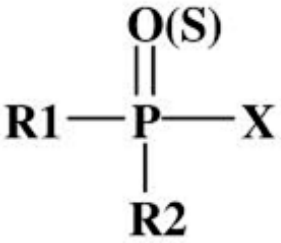
Phophorothioate compounds (P=S) need bio-activation to their phosphate analogues (P=O) in order to become biologically active (Sidell and Borak, 1992; Vale, 1998; Lotti, 2001). Biologically active phosphate (P=O) compounds show variability of action depending on the specific subunits occurring as R_1, R_2 and X (subgroups) (Sidell and Borak, 1992) (Fig 1.6). In the reaction step of phosphorylation and inactivation of the AChE molecule, the X moiety is displaced from the phosphorus atom (OP compound) by a serine hydroxyl group (AChE molecule). The kinetics of this reaction is determined by the X moiety (leaving group) (Sidell and Borak, 1992).

Reactivation of the inhibited AChE enzyme can be enhanced by oxime antidotes, such a pralidoxime or obidoxime. The OP molecule binds to the esteric site of the AChE enzyme. Oximes, first bind to the anionic site of the enzyme, followed by binding to the OP molecule. This pralidoxime-OP binding results in conformational changes to the OP molecule, thus leading to unbinding of the OP-AChE. This frees the enzyme esteric site, allowing the capacity to hydrolyse ACh molecules to recover.

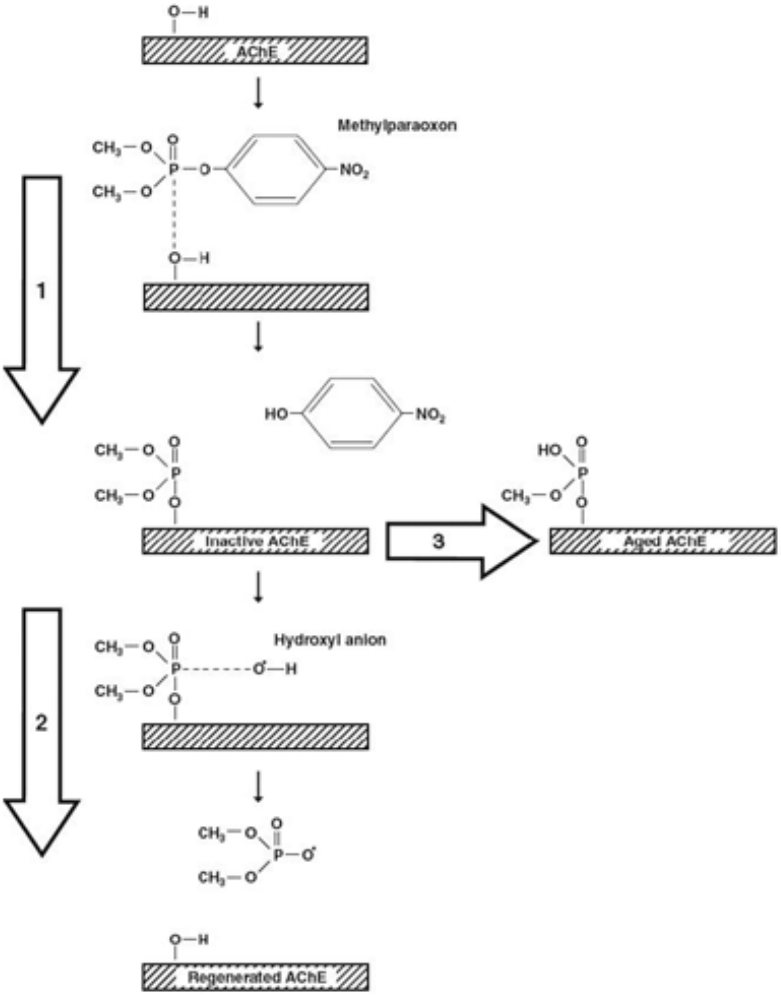
As a consequence of AChE enzyme inhibition, ACh is accumulated in the synaptic cleft. This results in persistent activation of acetylcholine receptors (AChRs) in the post synaptic membrane. Furthermore, cholinesterases inhibition increases the concentration of ACh left to escape by diffusion from the synaptic cleft (Katz and Miledi, 1973).

Fig 1.6: Pharmacology of OP action. A: General structure of organophosphate compounds comprising of R^{1-2} groups (hydrogen, alkyl, aryl and others, alkoxy and amino) and X which is a dissociable (leaving) group (halogens, cynos, alkylthio group, rest of inorganic or organic acid), B; steps of inhibition, regeneration and aging of AChE enzyme by organophosphorus compound. (Fig 1.5B was adopted from (Eddleston et al., 2002))

A


O(S)
R1
P
X
R2

B


AChE
Methylparaoxon
1
Inactive AChE
3
Aged AChE
Hydroxyl anion
2
Regenerated AChE

1.4.3 Consequential effects of OP and non-OP inhibition at NMJ

Acute and chronic effects of competitive and non-competitive inhibition of AChE have been studied widely in both clinical and experimental studies.

Electrophysiological examination is one of the most important parameters of clinical examination in diagnosing effects of toxicity on the peripheral nervous system (NMJ) in pesticide-poisoned patients. Normally repetitive stimulation produces no change in amplitude of compound muscle action potentials (CMAP) during a train of stimuli up to 30Hz (van Dijk et al., 2000). However at higher frequencies (>30Hz) an incremented pattern in the amplitude of CMAPs (pseudofacilitation) is observed (van Dijk et al., 2000; Jayawardane et al., 2009). Irregular patterns of CMAPs occur with repetitive stimulation following cutaneous exposure to OP compound. These include a decrement-increment response (CMAPs produced a reduction in amplitude followed by increase with in the train of stimuli), which is exacerbated by the administration of edrophonium (a reversible anticholinesterase inhibitor) (Maselli et al., 1986). Several other abnormal patterns of electromyographical recordings have been observed in different clinical studies with pesticide poisoned patients (Maselli et al., 1986; Besser et al., 1989a; De Bleecker, 1995; Jayawardane et al., 2008). These include: 1) decrement-increment, 2) combination of decrement-increment and repetitive fade, 3) severe decrement, and 4) progressive decrement. The severity of the abnormalities in CMAPs is increased with higher frequency stimulation (Jayawardane et al., 2008). It has also been reported that a decremented response in EMG with anticholinesterases can be attributed to desensitization of the post synaptic nAChRs.

Besides clinical studies, many experimental studies have examined the effects of AChE inhibition using OP and non-OP agents at NMJ, both *in-vivo* and *in-vitro*.

Primary post synaptic effects include changes in characteristics (rise time, amplitude and decay time) of both, MEPPs / MEPC and EPPs / EPCs.

Previous studies have shown an increase in MEPP amplitude with neostigmine (Boyd and Martin, 1956; Blaber and Christ, 1967), but subsequently decline to control value possibly due to desensitization or weak antagonism of AChR by the neurotransmitter (Eccles and Mac, 1949; Fatt, 1954). However, there is also evidence of unchanged MEPP amplitude with AChE inhibition in paraoxon (Laskowski and Dettbarn, 1979). On the other hand, lower concentrations such as 10^{-9} - 10^{-7}M neostigmine, ambenonium and edrophonium increase amplitude of EPPs by 0.5 - 2 fold (Blaber and Christ, 1967) while higher concentrations of

neostigmine, physostigmine and edrophonium (>10μM) depress the increment of EPC amplitude (Albuquerque et al., 1988).

Some studies observed 0.9 fold increment in MEPP rise time with neostigmine and ambinonium (1-10 μM) (Blaber and Christ, 1967; Tiedt et al., 1978); however the same concentration of pyridostigmine shows no significant change in MEPC rise time (Pascuzzo et al., 1984).

The most prominent effect of anticholinesterase treatment (carbamates and organophosphates) is a significant increase in the half decay time of the MEPP (Blaber and Christ, 1967; Laskowski and Dettbarn, 1979; Bois et al., 1980) and MEPC (Miledi et al., 1984) and EPP (Eccles et al., 1942; Eccles and Mac, 1949; Blaber and Christ, 1967; Katz and Miledi, 1975). Not only prolongation of the time course of EPP but also it's underlying EPC increased decay time with anticholinesterase treatment (Kordas, 1977). Treatment with anticholinesterase dramatically prolongs the time course of synaptic current decay due to lateral spread by diffusion of non-hydrolysed ACh which then repetitively binds to AChRs before removal from the synaptic cleft by diffusion (Katz and Miledi, 1973; Hartzell et al., 1975; Morrison, 1977).

There have been several reports on anticholinesterases binding to molecules at NMJ other than AChE. These molecules mainly include AChRs resulting in changed gating kinetics of the receptors (Kuba et al., 1974; Maleque et al., 1982; Akaike et al., 1984; Albuquerque et al., 1984; Pascuzzo et al., 1984; Albuquerque et al., 1985).

The occurrence of receptor desensitization with acetylcholinesterase inhibition had been frequently reported (Katz and Miledi, 1977; Wray, 1981; Akaike et al., 1984; Albuquerque et al., 1984; Shaw et al., 1985; Sherby et al., 1985) including evidence of NMJ transmission failure via desensitization of the post synaptic nicotinic receptors (Katz and Thesleff, 1957b; Thesleff, 1959; Katz and Miledi, 1973; Akasu and Karczmar, 1980; Magleby and Pallotta, 1981; Giniatullin et al., 1997; Giniatullin and Magazanik, 1998).

Anticholinesterase effects on presynaptic nerve terminal can be detected mainly with changes in spontaneous transmitter release (MEPP/MEPC frequency) and evoked transmitter release (quantal content).

There are limited reports providing evidence of changes in spontaneous transmitter release with anticholinesterase treatment. Prostigmine treated frog muscle (Fatt and Katz, 1951) or

rat diaphragm (Liley, 1956) showed no change in spontaneous transmitter release. On the other hand some studies have reported that smaller concentrations (μM) of anticholinesterase stimulate spontaneous release (Boyd and Martin, 1956; Blaber and Christ, 1967; Laskowski and Dettbarn, 1975; Bois et al., 1980), while reduction of rate the spontaneous release could be obtained at higher concentrations of prostigmine (Boyd and Martin, 1956; Duncan and Publicover, 1979) perhaps due to its curarizing action (blockage of postsynaptic nAChRs) (Eccles and Mac, 1949; Fatt, 1954).

It has also been shown that this increment of MEPP frequency that occurs via AChE inhibition is unlikely to be direct action of the drug on presynaptic terminals, as the MEPP frequency increment was reversed using an AChE reactivator pralidoxime chloride (Laskowski and Dettbarn, 1979). Moreover increased MEPP frequency also reversed with reduced Ca^{2+} and increased Mg^{2+} (Laskowski and Dettbarn, 1975) suggesting anticholinesterases lead to indirect effects on exocytosis, for example via the excess ACh activity presynaptic receptors, or as a consequence of depolarization of the terminal in response to additional efflux of K^+ via postsynaptic AChR when AChE is inhibited.

Alterations in presynaptic ion currents with anticholinesterases were also observed in several studies. It has been shown that neostigmine depresses the amplitude of K^+ currents in the perineural waveforms. This might account for the ability of neostigmine to increase the quantal content of the endplate potential (Braga et al., 1993). These authors also reported that anticholinesterase has a direct action on motor nerve terminals to block the delayed rectifier K^+ channels and enhance transmitter release. However, some reports shown that ACh itself suppresses delayed rectifier K^+ channels (Hevron et al., 1986; Shakiryanova et al., 1994). It was suggested that this modulation may be significant in cases of acetylcholinesterase inhibition, accounting for nerve terminal membrane hypersensitivity (Meir et al., 1999).

There is also evidence on dose dependent changes in presynaptic ultrastructure with AChE inhibition (Hudson et al., 1978). The authors reported the presence of vesicles which were two or more times the average diameter of synaptic vesicles, as were occasional vesicles with denser than normal content along with an irregularities present in the membrane recycling / vesicle formation system in acute and sub-acute anticholinesterase treated (*in-vivo*) preparations. Subsequent reports also further demonstrated alterations in the synaptic vesicles including increased number of coated vesicles with in vivo administration of paraoxon (Laskowski et al., 1977).

Spontaneous and stimulus induced repetitive firing in both nerve and muscle is another frequently reported feature of anticholinesterase activity, thought to be due to repeated action of ACh on its receptors (Morrison, 1977). Single stimuli induced repetitive muscle action potentials (RMAP) in anticholinesterase treated preparations were observed in many studies (Clark et al., 1983, 1984; Maselli and Soliven, 1991). These RMAPs are also responsible for repeated twitch potentiation triggered by single stimulus. It has been suggested that possible mechanisms of RMAP include persistent depolarization due to prolonged or repetitive EPP or both (Clark et al., 1984). In addition to repetitive firing, occurrences of spontaneous and evoked antidromic activity with anticholinesterases were also observed (Werner, 1960a, b; Laskowski and Dettbarn, 1975). The spontaneous muscle fasciculation and twitch potentiation produced by paraoxon (Barnes and Duff, 1953) and DFP (Van Der Meer and Meeter, 1956) in rat diaphragm preparations are thought to be due to antidromic action potentials being initiated at one nerve terminal and transmitted to other terminals of the same motor unit, thus giving rise to repetitive action potentials in its muscle fibres (Webb and Bowman, 1974). It is also suggested that these stimuli induced antidromic back firing with anticholinesterases, which impairs neuromuscular transmission of subsequent stimuli (Besser et al., 1992). These previous reports may explain the fasciculation and impaired neuromuscular transmission following pesticide poisoning.

Some of the studies have also examined the denervation or paralysis response with anticholinesterase treated preparations. Reported observations included physiological (decreased membrane potential with increase half decay time, slow rise time, low frequency in MEPP) (Meshul et al., 1985; Kawabuchi et al., 1991) and morphological (disruption of organelles in the axonal terminal and regional or total withdrawal of the nerve from the post synaptic folds) alterations in both acute and sub-acute exposure of anticholinesterases (Hudson et al., 1986). These studies suggest that mitochondrial and vesicular abnormalities were due to unusual level of mitochondrial activity and or an irregularity in the membrane recycling / vesicular formation system. Further reports shows sub lethal doses of sarin induced non Wallerian type (localized) axonal degenerative changes in the neuromuscular junction followed by subsequent sprouting with time (Kawabuchi et al., 1991). These authors showed that presence of MEPP at 800-2500µm away from endplate 5 days post toxicity compared to no MEPP at control samples (>600µm). This MEPP recording further indicated that slow rise time with longer half decay time and irregularity in shape. Degenerative ultra-structural changes with paraoxon included mitochondrial disruption, alterations in synaptic vesicles and altered cytoarchitectural organization of the post synaptic segment of the

neuromuscular junction (Laskowski et al., 1975, 1977). These effects were visible as early as 30 minutes after injection of the drug *in-vivo*.

These previous observations suggested that anticholinesterases have multiple effects on synaptic transmission at the NMJ, both conventionally (inhibition of AChE, thus repeated binding of ACh on AChRs) and non-conventionally (direct action of ACh on AChRs and presynaptic terminal due to increase concentration of ACh in the synaptic cleft). However, the contribution of anticholinesterase activity towards neuromuscular transmission failure reported in pesticide poisoned human patients is still debatable, because none of the reports show direct evidence of delayed muscle weakness (delayed synaptic transmission failure), as occurs in IMS.

1.4.4 Relationship of OP AChE toxicity to OP pesticide toxicity and IMS

Some studies have looked at the possibility of NMJ synaptic transmission block being due to AChE inhibition.

Acetylcholine can also act as a weak antagonist for ACh receptors due to its prolonged activation which cause desensitization or depolarizing neuromuscular block. Earlier it was noted a depression in EPPs amplitudes occurs with a wide range of anticholinesterases (Eccles and Mac, 1949). These were then identified as depolarizing effects (Nastuk and Alexander, 1954; Katz and Thesleff, 1957a; Blaber and Christ, 1967). It was reported that occurrence of cumulative depolarization of the endplate with repetitive nerve stimulation in the presence of anticholinesterase is partially due to accumulated residue of ACh which continued to act during the slow depolarization (Katz and Miledi, 1975) and stabilization of open ionic channels in the endplate membrane persisting after the transmitter has diffused away (Kuba et al., 1974). Evidence of depolarizing block with ambenonium and methoxyambenonium has been also reported (Blaber, 1960). Irreversible neuromuscular block was also detected with millimolar concentrations of paraoxon (Laskowski and Dettbarn, 1979).

It has also been observed that anticholinesterases induce an accumulative depolarization with high frequency trains of stimuli (Chang et al., 1986). It was hypothesised that this could be due to regenerative Ca^{2+}-spike which results in explosive release of ACh. Further evidence was provided on mechanisms for evoking regenerative release involving ACh receptors and Ca^{2+} channels which are different from these involved in the normal quantal release of ACh (Hong and Chang, 1989).

Some reports demonstrated that only a fraction of all AChE sites at a cholinergic junction are involved in the hydrolysis of ACh released from the nerve terminal (Koelle and Steiner, 1956; Mc and Koelle, 1959).

Tetanic fade (decrease in amplitude of the tension response or inability to sustained fused tetanic responce) in high frequency repetitive stimulation produced in the presence of OP and non-OP anticholinesterases has been reported frequently (Berry and Evans, 1951; Barnes and Duff, 1953; Van Der Meer and Meeter, 1956; Fleisher et al., 1960; Heffron and Hobbiger, 1979). It was shown that the tetanic fade caused by the anticholinesterase drugs are a consequence of depolarizing block through accumulating ACh (Blaber and Bowman, 1963).

The comparison of AChE activity and high frequency indirect stimulation has revealed that considerable reduction in functional AChE activity is required before the diaphragm loses its ability to respond with a sustained tetanus (Heffron and Hobbiger, 1979). These authors also stated that minimum (critical) level of functional AChE activity required for a normal tetanic response is directly related to the frequency of stimulation and once the functional AChE has been reduced to the critical level a very small further reduction leads to complete tetanic fade. In contrast, some reports demonstrate that fade of tetanic contraction caused by anticholinesterase is explained by the inactivation of sodium channels in the area surrounding the endplate, but that sustained fade is due to a decrease in transmitter release (Chang et al., 1986). There has also been speculation on a pre-junctional mechanism which causes endplate current train rundown and tetanic fade (Gibb and Marshall, 1987). In conjunction to the above assumption, it is observed that choline, hexamethonium and tubocurarine reduced both tetanic fade and endplate potential run-down caused by neostigmine, despite the fact that they themselves also induced these two effects. The mutual reversal by neostigmine and AChR antagonist of endplate potential run-down may implicate the presence of a positive (physiological increase of ACh to overcome high demand) and a negative (pharmacological decrease exert by the accumulated ACh) feedback regulation for evoked transmitter release via nicotinic AChR in the mammalian motor nerve, depending on the concentration of ACh within the synaptic cleft (Chang et al., 1988).

1.4.5 Efficacy, potency and mechanism of dimethoate/omethoate action

As the toxicity of dimethoate in animal depends on its conversion to oxon analogue, omethoate, it has been shown that omethoate has 10^5 times more potent effects than that of

dimethoate (Lucier and Menzer, 1970). Phenobarbital is an inducer of hepatic microsomal enzyme (Which convert dimethoate to omethoate) (Vardanis, 1966); pre-treatment of mice with phenobarbital leads to a significant increase in dimethoate toxicity (Menzer and Best, 1968). On the other hand, pre-treatment with sesamex (inhibitor of hepatic microsomal oxidation) did not reduce the dimethoate toxicity; as sesamex inhibit the detoxification pathways of both dimethoate and omethoate (Lucier and Menzer, 1970).

1.5 Organic solvent toxicity

Use of organic solvents has become increasingly prevalent due to their importance in the industrial use. There are many types of organic solvents available. Toxicity of these organic solvents arise depending on the mechanism of action (which is usually related to their structure) and the amount or dose of exposure of particular solvent.

1.5.1 Clinical syndromes

Among the many clinical symptoms caused by organic solvent toxicity in humans, neurotoxicity is most prevalent (White and Proctor, 1997). Depending on the exposure (large acute exposure or small chronic exposure) these compounds cause either temporary or permenent damage to CNS and PNS. CNS depression and psychomotor or attentional deficits along with the symptoms of fatigue, irritability, confusion, or depression, and memory difficulties may present when CNS is affected, while PNS symptoms include gradual onset intermittent tingling and numbness, with progression to an inability to perceive sensation and muscle weakness (White and Proctor, 1997). Diagnostic test indicate neurophysiological and neuropsychological functions used to diagnose clinical syndrome in these patients (White and Proctor, 1997).

1.5.2 Effects of organic solvents on NMJ

The effects of cyclohexanone and cyclohexanol on synaptic transmission are unknown and neither of them have been studied at the NMJ. However, it is noteworthy that the cyclohexanol analogue, 2-(4-benzylpiperidino) cyclohexanol (vesamicol), inhibits the vesicular loading of ACh molecules at the nerve terminal (Prior et al., 1992) causing a reduction in the quantum size. The effects of aliphatic alcohols on neuromuscular transmission have been studied. For instance, it was shown that ethanol reduces direct and indirect twitch and tetanic contractions by depressing NMJ transmission (Wali and Hayter, 1988). On the other hand, Searl and Silinsky (Searl and Silinsky, 2010b) demonstrated that

ethanol enhances synaptic transmission through post synaptic mechanisms, i.e. increasing the ACh channel open time. There have been some studies of the effects of ketones on neuromuscular transmission. For instance, Okada (1967) described effects of acetone on NMJ, showing a dose dependence of MEPP frequency and EPP decay; while Larsen et al (1996) shows that acetone has blocking effects on muscle Na^+ currents. Other studies of organic solvents include effects of dimethyl sulfoxide (DMSO) which also produces dose dependent effects on the NMJ including AChE inhibition (McLarnon et al., 1986). It was shown that irreversible uncoupling of the mechanical activity of the muscle produced by the glycerol treated nerve muscle preparations is through the alterations in the EPP amplitude (Sosa and Zengel, 1993).

Sumarry;

Pesticide poisoning leads to about 250,000 – 370,000 mortalities every year, and is responsible for about one third of global suicides. Understanding this is important in view of the major significance of pesticides, including Dimethoate EC, on human health, particulary in developing Asia, where their use remains widespread; and where suicides, or severe and debilitating life-threatening illness, including IMS, are a major health problem and a burden to hospital services. IMS, in particular, is poorly understood and there is no effective treatment except artificial ventilation, Understanding the mechanisms of action of the constituents of pesticides like Demothoate EC and their role in causing the paralysis that is characteristic of IMS could lead to the discovery and development of effective pharmacological treatment; relieving the burden of health care in rural communities in Asia and potentially saving thousands of lives annually. Understanding the mechansisms could also lead to deeper biological insight into the function of several components of the neuromuscular system and perhaps to other paralysing disease in which neuromuscular function is compromised.

1.6 Aims and objectives

1.6.1 Overall Aim

In order to understand the mechanisms triggering the respiratory failure due to neuromuscular transmission block during the intermediate syndrome of OP poisoned patients, we must first address the effects of these pesticide and their metabolites, either alone or in combination, on neuromuscular transmission. As I have discussed above anticholinesterases appear to have both pre and post synaptic effects on neuromuscular

synaptic transmission. However, the causes of delayed muscle weakness and paralysis due to this anticholinesterase are still unknown. Furthermore, direct evidence of effects of the solvent used in industrial pesticides and their metabolites on NMJ synaptic transmission is missing.

1.6.2 Detailed hypotheses

My overall aim in this thesis was to test the hypothesis that the delayed respiratory failure after ingestion of pesticide is due to failure of neuromuscular transmission; and that this is a consequence of initial excitotoxicity, resulting from inhibition of acetylcholinesterase. To test this overall hypothesis, I have investigated the effects of components of one particular commercial pesticide: dimethoate EC.

As I have mentioned earlier, Dimethoate EC is a moderately toxic pesticide according to the WHO classifications. Moreover, due to high toxicity in class 1 OP pesticides, the Food and Agriculture Organization (FAO) has advised withdrawal of more class I pesticides from agricultural practice (FAO, 2002). Therefore, it is highly likely there will be an increase in use of Dimethoate Ec40 in agricultural practice world wide. Furthermore, Dimethoate toxicity in humans is responsible for higher mortality rates with increased cardiotoxicity and development of IMS (Dawson et al., 2010). Thus, use of dimethoate in my experiments is appropriate in order to understand the mechanism of IMS and development of new therapeutic regimes.

My detailed hypotheses were that:

1. The initial, acute syndrome is caused by the anticholinesterase effects of dimethoate at the neuromuscular junction (NMJ).
2. Metabolic conversion of dimethoate and cyclohexanone to omethoate and cyclohexanol, respectively, contribute to acute pesticide toxicity at the NMJ.
3. Transmission failure in IMS is due to the persistent combined effects of dimethoate, cyclohexanone, omethoate and cyclohexanol on neuromuscular transmission

1.6.3 Three objectives

The objectives in the three Results chapters were therefore to examine the main predictions from the above hypotheses, as follows:

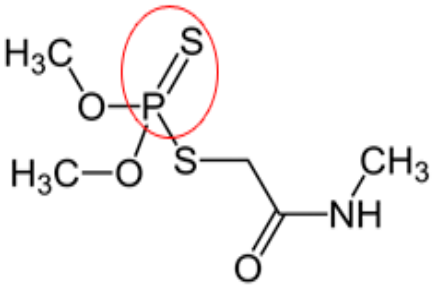
i. First I investigated how the pesticide, its ingredients dimethoate and cyclohexanone, and their metabolites omethoate and cyclohexanol (Fig 1.7), respectively, affected synaptic transmission at the NMJ. This was done by examining cellular (intracellular recordings) and functional (isometric tension) characteristics of neuromuscular synaptic transmission with pesticide components, either alone or in combination (Chapter 3). The results show that omethoate and cyclohexanol have potent, additive effects in prolonging synaptic potentials and isometric contractile responses that exceed those of dimethoate or cyclohexanone.

ii. Next, I investigated further the mechanisms by which omethoate and cyclohexanol interfered with neuromuscular transmission, by measuring passive membrane properties and synaptic currents under voltage clamp (Chapter 4). I found evidence for complex presynaptic and postsynaptic mechanisms in addition to effects attributable to inhibition of AChE.

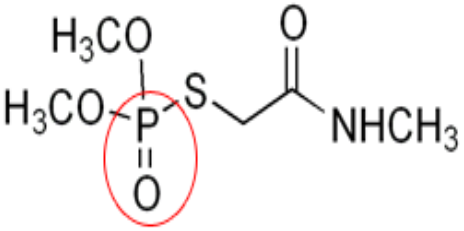
iii. Finally, I attempted to model the neuromuscular transmission failure in IMS and to verify whether it is due to the persistent combined effects of omethoate and cyclohexanol on neuromuscular transmission. This was accomplished using a novel *ex vivo* assay. I found that a cocktail of dimethoate, omethoate, cyclohexanone and cyclohexanol impaired synaptic transmission and enhanced synaptic degeneration ex vivo. I extended these findings by demonstrating additional, use-dependent deterioration of transmission following additional, acute stimulation in the presence of pesticide ingredients and their metabolites (Chapter 5).

Fig 1.7: Structural formulae of the compounds used in this thesis. The P=S in dimethoate (red circle) is oxygenated in the liver to P=O in omethoate (red circle), by the enzyme complex cytochrome P450. Cyclohexanone is converted in to cyclohexanol in the stomach and small intestine by reverse activation of alcohol dehydrogenase. The normal substrate for this enzyme is ethanol, which is reduced to acetone. Neostigmine is a quaternary nitrogen compound which inhibits AChE activity, but has no organic phosphate group.

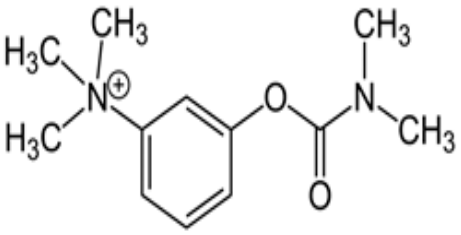
Dimethoate



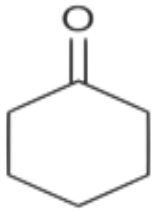
Omethoate



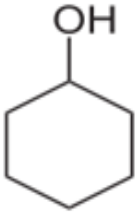
Neostigmine



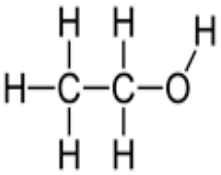
Cyclohexanone



Cyclohexanol



Ethanol



1.6.4 Preview of conclusions

Taking all the data from my studies together, I conclude that failure of neuromuscular transmission induced by pesticide ingestion cannot be explained solely by inhibition of acetylcholinesterase by either dimethoate or omethoate. Rather, the metabolic breakdown products (both omethoate and cyclohexanol) exert an additive or synergistic, harmful combination of presynaptic and postsynaptic effects. Interestingly, in addition to its postsynaptic effects, cyclohexanol appears to show similar presynaptic effects to vesamicol on neuromuscular transmission, and also to affect presynaptic membrane currents. Finally, the results of my study utilising an isolated *ex-vivo* assay suggest that prolonged exposure to both the ingredients and their metabolites may be required to produce IMS. This novel assay may also constitute a fruitful method for evaluating pesticide toxicity from longer duration exposures and its modifiers.

These findings, and further understanding of the mechanisms of synaptic toxicity that they may lead to, could facilitate the development of better therapeutics and more effective management of pesticide poisoned human patients, possibly saving thousands of lives each year.

Chapter 2: Materials and methods

Methods outlined below are common to some of the experiments reported Chapters 3-5. Methods specific to experiments reported in those Chapters are described in their Methods sections.

2.1. Animals

2.1.1 Animal care and housing

Mice were maintained in the facilities at the University of Edinburgh in accordance with UK Home Office regulations (United Kingdom Animals (Scientific Procedures) Act, 1986). All mice used were housed in standard light (12h light 12h darkness) and temperature conditions and in cages of 6 or fewer. Mice had access to food and water ad libitum.

Age matched male and female C57/Bl6 (6 – 12 weeks) mice were used in most experiments. In some experiments I utilized C57 *Wld*^{s} and thy1.2-YFP16 *Wld*^{s} (6 – 8 weeks) (6 – 8 weeks) mice (Wong et al., 2009).

2.1.2 Animal sacrifice

All the mice were killed by cervical dislocation, according to the UK Home Office regulations, Schedule 1.

2.2 Composition of the salines

Hepes-buffered mammalian physiological Saline (MPS: 137mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM Hepes, 5.6mM Glucose, equilibrated by bubbling with air, supplied by a domestic aquarium pump) was prepared at room temperature (20 – 25 ^{0} C) in advance of the animal sacrifice. The pH of the solution was measured using a standard pH meter (Sartorius pH meter PB-11) and adjusted to pH 7.2 - 7.4 using 1M NaOH or 1M HCl.

2.3 Muscle disections

2.3.1 Flexor digitorum brevis (FDB) muscle preparation

Directly after sacrifice, hind limbs were amputated and skin was stripped. Then limbs were placed in fresh MPS for dissection. Each of the legs was pinned (minutien pins) in a Sylgard-lined Petri dish. The FDB muscle along with the tibial nerve was dissected and nerve-muscle preparation then transferred back to MPS until commencement of the physiological recording. The remaining part of the leg was discarded.

According to Bekoff and Betz (1977b; Bekoff and Betz, 1977a), the short length of FDB muscle fibres in rats suggested that membrane electrical parameters are equivalent to a short cable model with open circuit ends. The most important practical consequence of this is that FDB muscle fibres (which are even shorter in mice, see (Gillespie and Ribchester, 1988) are isopotential. Thus, endplate depolarization spreads rapidly without decrement throughout the muscle fibre.

It was shown that rat FDB muscle fibres are isopotential along their length: thus, current generated from a source located at any point along the fibre (including the NMJ) can be faithfully recorded by a microelectrode located anywhere else in the fibre (Bekoff and Betz, 1977b; Bekoff and Betz, 1977a). Mouse FDB muscle fibres are also short (<500μM) (Gillespie and Ribchester, 1988; Ribchester et al., 1995; Gillingwater et al., 2002; Ribchester et al., 2004) and therefore could be expected to behave in a similar manner to that of rat muscle fibres. These properties demonstrate that FDB is a particularly suitable muscle for routine electrophysiological examinations of neuromuscular transmission.

2.3.2 Trangularis sterni (TS) muscle preparation

Directly after sacrifice, the mouse was pinned on a cork board. Thoracic skin was removed and the ribcage was carefully dissected out, and then transferred to Hepes MPS at room temperature.

The ribcage was then halved symmetrically along the sternum and one half was pinned on Sylgard lined Petri dish which contained Hepes MPS for dissection. The trangularis sterni muscle (single layer thin muscle) along with the corresponding intercostal nerves (McArdle et al., 1981) was exposed after careful removal of second to fifth ribs. Nerve-muscle preparations were then transferred back to MPS until commencement of the electrophysiological recording.

2.3.3 Lumbrical muscle dissection and staining

Lumbrical muscle dissection was carried along with the dissection of FDB nerve muscle preparations for *ex-vivo* culture assay (see above). After 24 hours of culture, the combined FDB and lumbrical nerve muscle preparation was then returned to a Petri dish containing MPS ± drug treatment. The lumbrical muscle was then carefully separated from the FDB muscle and the FDB was then used for physiological analysis. The lumbrical muscles were pinned out in a Sylgard lined Petri dish and submerged in MPS containing 5μgml^{-1}

tetramethylrhodamine-isothiocyanate (TRITC) α-bungarotoxin (Molecular Probes, USA) which labelled post synaptic acetylcholine receptors (AChRs). The preparation was then placed on a rocking platform (Stuart Scientific, UK) for 10 minutes covered in tin foil. The sample was washed three times with MPS for 10 minutes each, and then fixed by immersion in 4% paraformaldehyde (Electrone microscopy science, USA) for 15 minutes followed by two cycles of washing with MPS as previously. Fixed muscles were then mounted on glass slides in Vectashield (Vector laboratories, UK) and covered with a coverslip ready for fluorescence microscopy.

2.4 Drug and toxins

The following were used: μ-conotoxin (μ-CTX) (GIIIIB μ-CTX; Bachem AG, Switzerland), tetramethylrhodamine-isothiocyanate (TRITC) α-bungarotoxin (Molecular Probes, USA), d-tubocurarine (15mg/1.5ml, Evans medical LTD, England), neostigmine (2.5mg/ml, Phoenix Pharma LTD, Gloucester), dimethoate (Cheminova, Denmark), omethoate (Sigma aldridge, United kingdom), cyclohexanone (Sigma Aldridge, United kingdom), cyclohexanol (Sigma Aldridge, United kingdom).

2.5 Electrophysiological apparatus and intracellular recordings of MEPP/EPP

All experiments were done at room temperature (20 - 25°C).

2.5.1 EPP recording (Indirect stimulation)

Preparations were pre-treated with MPS containing 2.5 μM μ-CTX for 20 minutes or until twitch contractions had disappeared. μ-conotoxin is contained in the venom of the piscivorous marine snail, *Conus geographus L* (Cruz et al., 1985). This toxin blocks voltage gated muscle sodium channels (Na_v 1.4) (Li and Tomaselli, 2004), thereby abolishing muscle action potentials, while having no discernible effect on nerve sodium channels (Braga et al., 1992; McIntosh and Jones, 2001; Gillingwater et al., 2002; Ribchester et al., 2004).

After incubation with μ-conotoxin, the preparation was pinned in a Sylgard-lined chamber, approximate volume 10 ml, filled with MPS. The nerve was positioned in glass suction electrode and stimulated via a Digitimer DS2 isolated constant voltage stimulator driven by a Digitimer D4030 programmer. Microelectrodes (Harvard apparatus / glass capillary tube, GC150F-15, 1.5 O.D. * 0.86 I.D.) for intracellular recordings were pulled using a Flaming / Brown micropipette puller (P87 Sutter instrument. USA). Electrodes were back filled with 4 – 5M potassium acetate and had microelectrode tip resistances of 30 – 50 MΩ. Membrane

potentials were recorded using an HS-2A (*0.1LU) head stage mounted on Leica micromanipulator and connected to Axoclamp 2B amplifier (Axon instrument, USA). The reference electrode was a silver wire connected to an Ag/AgCl pellet immersed in the bathing medium and connected to the signal ground of the head-stage. Signals were amplified using Neurolog AC/DC amplifier (neurology NL 106), low pass filtered at 3 KHz (neurology NL 92) and then passed through a Humbug filter (Quest scientific, Canada) to eliminate 50 Hz main interference. Signals were digitized (50KHz) in to a personal computer (DELL., USA) via Micro 1401 mk II (Cambridge Electronic Design Limited., England) interface. EPP recordings were analysed using WinWCP software (Dr John Dempster, Strathclyde Electrophysiological Software, University of Strathclyde, UK) for several characteristics (Fig 2:1). These measurements include latency, peak amplitude, rise time, half decay time and quantal content.

Before each drug treatment, 6 -12 EPP recordings were obtained from randomly selected fibres. The preparation was stimulated with a trains-of-four nerve stimuli, delivered at 33Hz (interval 30ms) with an interval of 5s between each stimulus train. The trains were repeated up to 30 times. The preparation was then incubated for 20 minutes with respective drug treatment before commencing further recording.

2.5.2 Recording & analysis of MEPPs

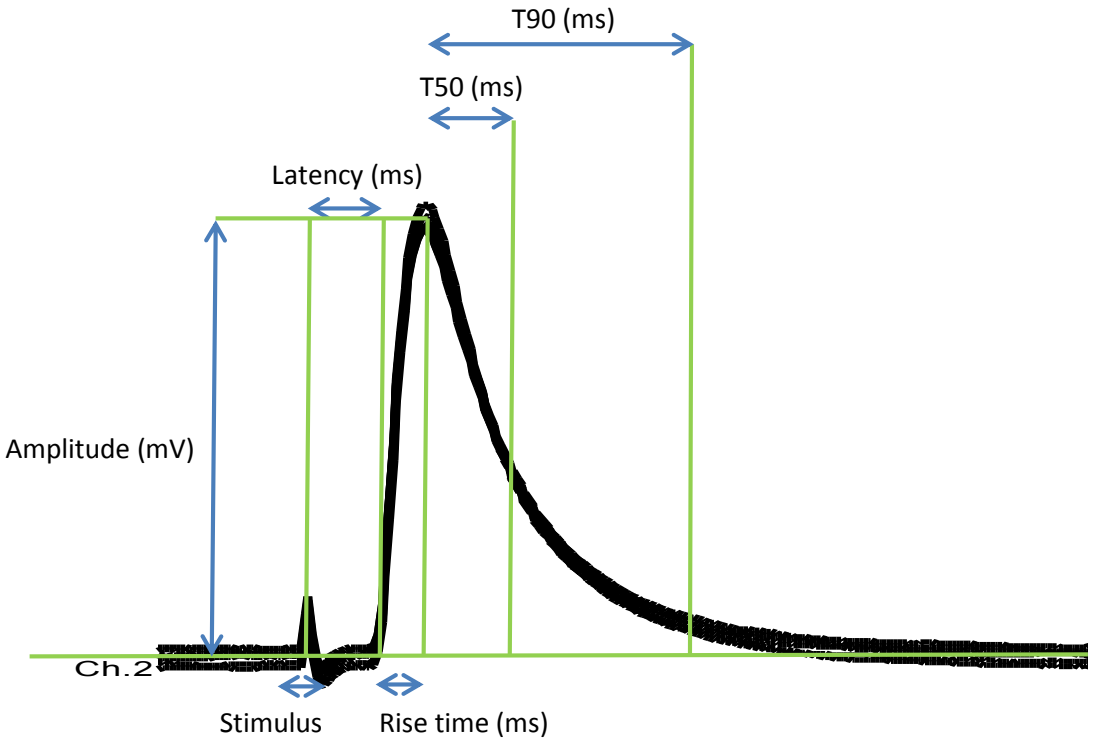
Spontaneous activity was recorded using direct couple (DC) condition for 60 – 100s at each fibre with a steady membrane potential at -60 to -70mV. Recordings were discarded if the resting membrane potential had drifted more than 20mV difference from the original value. MEPPs were recorded via a CED 1401 Micro mk II using Spike– 2 software (Cambridge Electronic Design, UK) digitized at 50KHz (20µsec/cycle). Analysis of the recordings was carried out with Mininalysis software (Synaptosoft, USA). The specific data files were first converted to Axon binary files (ABF) and an amplitude correction factor of 0.2 was applied to correctly calibrate the data recordings. Then the data were processed to detect characteristic MEPP parameters such as rise time, amplitude, decay time and inter event-interval-frequency.

2.6 General statistical method for data analysis and composition of group data

All the data represented as mean ± SEM, unless otherwise stated. “N” numbers refer to as number of muscles tested. “P” values were represented as conventional stat ($P < 0.05 = *$). The Primary statistical data analysis was performed using Graph pad Prism software

(GraphPad Software Inc, USA). Multiple groups were compared using analysis of variance (ANOVA) ± post-test (Bonferoni). Unpaired t-tests were used where applicable to comparison of two groups.

Fig 2.1: Analysis of the endplate potential parameters. End plate potential properties were measured using WinWCP software. Time of the stimulation was displayed by the stimulus artefact appears at the beginning of the trace. The measured parameters include; peak amplitude of the EPP, latency (time between the stimulus and the beginning of the response) and rise time of the peak. Decay time was analysed as T50, i.e. using WinWCP the time for the EPP amplitude to decay from 90% to 50% of its original value.


T90 (ms)
T50 (ms)
Latency (ms)
Amplitude (mV)
Ch.2
Stimulus
Rise time (ms)

Chapter 03: Effects of Dimethoate EC pesticide, its constituents and metabolites on neuromuscular transmission and function

3.1 Background

Effects of anticholinesterases on neuromuscular synaptic transmission have been studied extensively since the early 20th century. For instance, Brown et al (Brown et al., 1936; Bacq and Brown, 1937; Brown, 1937) made myogenic recordings from anaesthetized cat muscles and reported repetitive contraction, resembling tetani, in response to single nerve stimuli following intravenous injections of eserine (presently known as physostigmine), an extract from the calabar bean. Eccles et al (1942) used extracellular field potential recordings to measure EPPs and reported that the principle effect of eserine on cat and frog muscles was "lengthening of the action of the neuromuscular transmitter, thus leading to prolonged junctional negativity with consequent catelectrotonic effects". Fatt and Katz (1951) and Del Castillo & Katz (1954) extended these studies using intracellular microelectrode recording to demonstrate the prolongation of spontaneous MEPPs and evoked EPPs by physostigmine at neuromuscular junctions in frog muscle. Further widening the use of anticholinesterases, Katz and Thesleff (1957a) used edrophonium and neostigmine to examine neuromuscular transmission in frog muscles, showing edrophonium potentiated the depolarizing effects of ACh. However potentiation of depolarizing effects was weak when preparations were pre-treated with neostigmine. Since these pioneering studies, there have been many reports describing effects of anticholinesterases on neuromuscular transmission, using intracellular microelectrode recording.

The use of OP compounds, such as sarin, in chemical warfare can be traced back to the 1930's. However, studies of their anticholinesterase effects on neuromuscular transmission became available only in the early 1950's (Berry and Evans, 1951; De Candole et al., 1953). OP also became increasingly used as pesticides from the middle of the 20th century: parathion was among the first marketed. However, detailed *in-vitro* electrophysiological analysis of the effects of the active ingredient of OP pesticides, such as paraoxon, only became available in the 1970's (Kuba et al., 1974; Laskowski and Dettbarn, 1975). Interestingly, OP compounds such as octamethyl pyrophosphoramide (OMPA) were also initially used as an anticholinesterse treatment for myasthenia gravis, as an alternative to neostigmine (Silvestri and Wolfe, 2012), but with adverse consequences of a "cholinergic crisis" that exacerbated the original condition in some cases.

Most of the available evidence concerning the effects of formulated pesticide (ie the commercial agricultural products) on NMJ synaptic transmission had been reported in the context of human clinical data (Jayawardane et al., 2008). For instance, electrophysiological

analysis of toxicity of pesticide containing the OP dimethoate initially focused on the anticholinesterase activity of intraperitoneally-injected pure compound (Dongren et al., 1999). Until recently, little consideration was given to the possible toxicity of the organic solvent in which the dimethoate is normally dissolved in the commercial pesticide, after it is ingested (Eddleston et al., 2012). This deficiency demonstrates a lack of knowledge and understanding of potential combinatorial actions of the components of pesticides on neuromuscular transmission, at molecular, cellular and overall functional levels.

As I introduced in Chapter 1, the pesticide known as dimethoate EC 40 comprises three main components; dimethoate (the "active ingredient"), cyclohexanone (an organic solvent), and an emulsifying agent that acts as a surfactant (unknown molecular identity, apparently a trade secret). When ingested, the known pesticide ingredients undergo metabolism in the intestine and liver. I have summarized in Chapter 1, the breakdown and excretion of dimethoate EC40 and its metabolites but the principle metabolic conversions I have focused on in this chapter are those from dimethoate to omethoate, and from cyclohexanone to cyclohexanol. The blood plasma of human patients poisoned with dimethoate EC contains all four compounds (Eddleston et al 2008) and this was emulated and reproduced in a pig model (Fig 1.2) (Eddleston el at 2012).

For the research described in the present Chapter, I set out to measure the effects on neuromuscular transmission of formulated pesticide and its OP and solvent components, singly and in combination. I used NMJ in isolated flexor digitorum brevis (FDB) muscles from mice as my experimental paradigm because FDB muscle fibres are short, isopotential and therefore readily accessible to intracellular recording and electrophysiological analysis of synaptic transmission, for instance in models of synaptic degeneration (Bekoff and Betz, 1977b; Ribchester et al., 1995; Gillingwater et al., 2002; Ribchester et al., 2004) (Brown et al 2014, in press).

First, I studied the effects of plasma obtained from minipigs poisoned with the formulated dimethoate EC pesticide, or with dimethoate alone, on spontaneous MEPPs and evoked EPPs, measured with intracellular microelectrodes. Second, I measured the effects of dimethoate, omethoate, cyclohexanone or cyclohexanol, (collectively referred to as DOCC) singly and in various combinations when added to Hepes-buffered mammalian physiological saline (HEPES-MPS) bathing the isolated nerve-muscle preparations. I constructed dose-response curves for the two most potent of these compounds: omethoate and cyclohexanol. Thirdly, I investigated the responses of NMJs in the presence of DOCC and its components

to repetitive, patterned nereve stimulation, in order to emulate respiratory activity, since the clinical signs and symptoms following pesticide ingestion suggest that both the acute cholinergic crisis and subsequent paralysis of muscle in IMS appear to be activity-dependent. Lastly, I determined the effects of omethoate and cyclohexanol on overall functional responses, measured from twitch and tetanic muscle contractions.

The data suggest that the metabolites of Dimethoate EC, specifically omethoate and cyclohexanol, potently interact to prolong EPPs but by different mechanisms. In combination, they produce profound adverse and synergistic effects on neuromuscular transmission and function.

3.2 Methods

The following methods are specific to this chapter. For general methods and chemicals, please refer to Chapter 2.

Base line recordings for all the experiments were carried out in Hepes MPS at room temperature.

3.2.1 Minipig plasma samples and intracellular recording (EPP/MEPP)

Plasma samples (arterial blood) were obtained from treated minipigs 10 – 12 hrs after administration of pesticide (2.5ml/kg), dimethoate (1g/kg) or saline (2.5ml/kg) by oral gavage. These plasma samples (2ml) were centrifuged at 1200 RPM for 2 minutes and supernatant was retained. First, baseline EPP / TOF and MEPP measurements from 10 fibres were obtained from μ-CTX pre-treated (as described in general methods) FDB nerve-muscle preparations. Further EPP or MEPP recordings of 20 - 30 fibres were made with treatment (≈ 2ml of respective plasma or volume of neostigmine / positive control to obtain 10μM final bath concentration) added to the recording chamber followed by a 20 minutes equilibration period, leaving a 10 minutes interval after every sixth fibre.

3.2.2 Intracellular recordings (EPP / MEPP) with purified pesticide ingredients and their metabolites

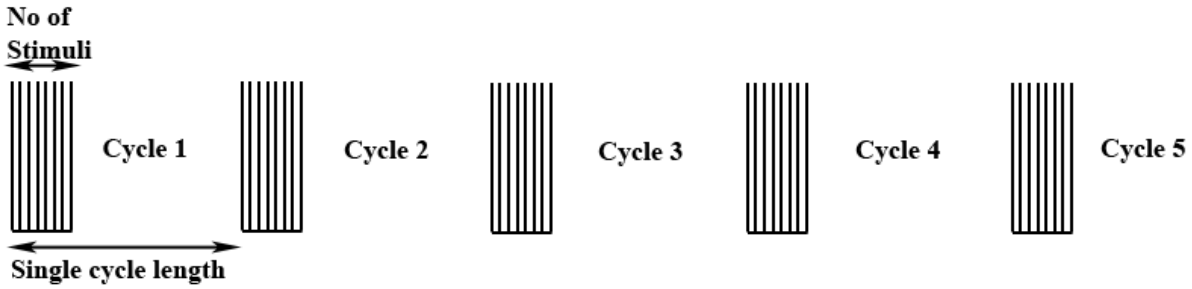
Baseline recording of EPPs and MEPPs were made from in 6 – 10 muscle fibres (μ-CTX pre-treated preparations) according to the stimulation protocol stated below (step 1 – step 10) (Fig 3.1). Each of the impaled muscle fibres was stimulated with the patterns described below. Test compounds were then added to the recording chamber to the final concentration required. After 20 minutes equilibration, intracellular recordings were made from further 6 – 10 muscle fibres with 10 minutes interval at each fibre recording. This was in order to mitigate presynaptic vesicular depletion and/or desensitized post synaptic receptors as a consequence of high frequency repetitive stimulation. Treatments included pure compounds of formulated pesticide dimethoate EC (i.e. dimethoate and cyclohexanone), either alone or in combinations with their metabolites (omethoate and cyclohexanol). Treatment concentrations for this experiment were adopted from Eddleston el al 2012, based on plasma concentrations in the Göttingen minipig model of OP-pesticide poisoning.

The stimulation protocols for individual recordings were included in the table below. Stimulation protocol (step 1 – 10) was repeated for every sampled muscle fibre.

Initial characterization					
	Step 1	Step 2	Step 3	Step 4	Step 5
No of cycles per recording	5	5	5	5	5
Stimulation frequency	**1Hz**	**2Hz**	**5Hz**	**10Hz**	**20Hz**
Single cycle length	7s	7s	7s	7s	7s
No of stimuli per cycle	4	4	10	10	10

Rhythmic pattern stimulation					
	Step 6	Step 7	Step 8	Step 9	Step 10
No of cycles per recording	5	5	5	5	5
Stimulation frequency	50Hz	50Hz	50Hz	50Hz	50Hz
Single cycle length	**5s**	**5s**	**5s**	**2.5s**	**2.5s**
No of stimuli per cycle	10	25	100	10	50

Fig 3.1: Schematic illustration of rhythmic patterned stimulation protocol. Note that number of stimuli per cycle changes from 10 to 100 and single cycle length was varied between 5s and 2.5s.


No of
Stimuli
Cycle 1
Cycle 2
Cycle 3
Cycle 4
Cycle 5
Single cycle length

Sampling protocol:

Intracellular recordings of MEPP and EPP were carried out as follows. First, 20s – 30s of MEPPs were recorded. Then from the same fibre, EPPs were recorded. Stimulation protocols were designed with sufficient interval between each cycle, to avoid fatiguing the muscle. This protocol was then followed by further 20s – 30s of MEPP recording. I then continued with the rhythmic patterned high frequency stimulation steps. Finally another 20s – 30s of MEPPs recorded on the same fibre, to observe whether there was any evidence for presynaptic modifications (e.g. synaptic vesicle depletion) following high frequency burst stimuli, in the presence of pesticide and their metabolites.

3.2.3 Sampling protocol for drug concentration curves

Cumulative drug dose-response data were acquired as follows. First, baseline EPP / TOF or MEPP measurements were obtained from 10 muscle fibres in μ-CTX pre-treated FDB nerve-muscle preparations (see above). Then the respective drugs were added to the bath to achieve the targeted drug concentration. After a 20 minutes equilibration, further EPP or MEPP recordings were made from 20 fibres, placing a 5 minutes interval after every fifth fibre. The protocol continued until data from all the accumulated drug concentrations had been obtained from the same muscle.

3.2.4 Measurements of AChE activity

The following procedure was carried out in New Jersey Medical School-Rutgers University, Newark, United States of America by Dr. V. Patel and Dr. L.G. Sultatos.

Hemidiaphragms were dissected, weighed, and homogenized in 9 volumes of 100 mM sodium phosphate buffer (pH 7.4). Production of thiocholine in the Ellman reaction was used to measure AChE activity. Briefly, a total of 1 ml of reaction volume containing 0.44 mM acetylthiocholine and 0.1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) was placed in a cuvette. The reaction was initiated by addition of 50 μl of muscle homogenate, and the increase in optical density at 412 nm (due to thiocholine production) was followed for 10 min in a Shimadzu UV2550 spectrophotometer (Kyoto, Japan). The slopes of optical density curves were determined by linear regression (Sigmaplot 8, Systat Software, Chicago, IL).

3.2.5 Isometric tension measurement recordings

Tension recordings were carried out using tibial nerve - FDB muscle preparations. Following dissection, the proximal tendon of the FDB muscle was attached to black braided 7.0 silk sutures less than 2cm in length, (Fine Science Tools, Germany) tied in a loop at the free end. The preparation was then pinned using its three distal tendons in a Sylgard-lined recording chamber filled with MPS. The threaded end was then attached to an Akers / Sensonor AE875 force transducer rigidly mounted on micromanipulator.

The force transducer was connected to a Neurolog NL106AC/DC amplifier via a Wheatstone bridge circuit powered by a rechargeable 6V battery, built in house. The muscle and the force transducer were aligned orthogonally. Resting length was adjusted to give a maximum evoked twitch response. Amplified signals were then fed into a personal computer (DELL., USA) via a CED Micro 1401 mk II interface (Cambridge Electronic Designs, UK). Both isometric twitch and tetanic tension were recorded using Spike–2 software (Cambridge Electronic Design, UK) digitized at 1 KHz (1ms/point). The preparation was then incubated for 20 minutes with respective drug treatments and the above stimulus protocol repeated to obtain the measurements of their effects.

For indirect tension measurements, the nerve was positioned in glass suction electrode and stimulated via a Digitimer DS2 isolated constant voltage stimulator connected to a Digitimer D4030 programmer. For direct stimulation two silver wires were positioned alongside the muscle. These electrodes were connected to the DS2 stimulator.

3.2.6 Sampling protocol for twitch / tension measurements

The following steps are the sampling protocol. First motor unit recruitment was measured in response to graded indirect stimulation. Base line recording of twitch and tetanic tension measurements in Hepes MPS were then obtained in response to 40 stimuli delivered at frequencies of 0.5Hz, 1Hz, 2Hz, 10Hz, 20Hz and 50Hz, with an interval of 2 minutes between each stimulus frequency. Drug treatment was then added to the recording chamber followed by a 20 minutes incubation period. During incubation, the muscle was continuously stimulated indirectly at 0.1 Hz and resulting twitch tensions were monitored and recorded. Further recordings of twitch and tetanic tension at different stimulation frequencies similar to base line recordings, were then obtained. Direct stimulation was carried out in Hepes MPS, containing d-tubocurarine (5μM) to abolish neuromuscular transmission, with pulses of 20 – 30V intensity and 1ms duration. Drug concentrations (dimethoate, omethoate,

cyclohexanone and cyclohexanol) for these experiments were adopted from Eddleston el al (2012) as for EPP recordings.

3.3 Results

In accordance with the overall hypothesis and predictions indicated in the General Introduction, my primary objective in these experiments was to establish how dimethoate EC pesticide, its dimethoate and cyclohexanone solvent ingredients, and their metabolites omethoate and cyclohexanol, respectively, affect neuromuscular transmission.

3.3.1 Pesticide plasma prolongs neuromuscular transmission

Electrophysiological analysis of trains-of-four EPP showed that control plasma (plasma from minipigs treated with saline) added to FDB muscles bathed in Hepes MPS did not alter EPP characteristics. In other words, there was no discernible effect of plasma alone on neuromuscular transmission (Fig 3.2).

Pesticide plasma samples appeared to prolong EPP time course demonstrating statistically significantly difference from control MPS (half decay time: Hepes MPS; 3.02±0.6ms, control plasma; 3.24±0.28ms, pesticide plasma; 5.04±0.8. mean ± SEM, ($P < 0.05$, Bonferroni's, "F = 8.08") (Fig 3.2, 3.3, 3.4). Interestingly, prolongation of the synaptic potentials was evident 2 – 4 hrs post treatment with pesticide plasma preparations, whereas neostigmine took less than 10mins to act and dimethoate plasma took about 30mins to act (Fig 3.5). However, neither half-decay time nor peak amplitude of MEPPs indicated any statistically significant alteration from the control MPS values (half decay time: Hepes MPS; 2.63±0.81ms, pesticide plasma; 2.87±0.65ms. mean ± SEM, $P > 0.05$, ANOVA) (Fig 3.8, fig 3.9). However, there was a significant increase in MEPP frequency with pesticide plasma treated samples compared to the Hepes MPS, suggesting a stimulation of spontaneous transmitter release from presynaptic motor nerve terminals (Hepes MPS; 0.68±0.34s^{-1}, dimethoate plasma; 0.53±0.2734s^{-1}, pesticide plasma; 1.27±1.2034s^{-1}, neostigmine; 0.66±0.4434s^{-1} mean ± SEM, $P < 0.05$, Bonferroni's, "F = 9.84") (Fig 3.9 C). These data suggests that other ingredients present in the pesticide plasma have additional effects on NMJ transmission. Based on the findings reported in pigs (Eddleston et al., 2012), I hypothesised this was likely to be the pesticide solvent, or its metabolites.

Dimethoate plasma prolonged EPP decay similar to the effect of neostigmine (see below), consistent with the anticholinesterase activity of dimethoate (and / or its metabolites, see below), 30 – 60 minutes after adding plasma to the recording chamber (Fig 3.2, 3.3, 3.4, 3.5). The half decay time of MEPPs and EPPs were increased compared to its control Hepes MPS samples (EPP half decay time: Hepes MPS; 3.02±0.6ms, control plasma; 3.24±0.2ms, dimethoate plasma; 5.4 ±1.05ms. mean ± SEM, $P < 0.05$, Bonferroni's, "F = 8.08") (MEPPs half decay time: Hepes MPS; 2.63±0.81ms, dimethoate plasma; 3.35±0.97ms. mean ± SEM, $P < 0.05$, Bonferroni's, "F = 13.30") (Fig 3.8, 3.9).

Neostigmine (which I employed as a positive control for anti-AChE activity), as expected, prolonged synaptic transmission, producing a significant increase in half decay time of both MEPPs (Hepes MPS; 2.63±0.81ms, neostigmine; 3.66±0.81ms. mean + SEM, $P < 0.05$, Bonferroni's, "F = 13.30") and EPPs (MPS; 3.21±0.75ms, control plasma; 3.24±0.28ms, neostigmine; 5.41±1.23ms, mean ± SEM, $P < 0.05$, Bonferroni's, "F = 8.08") (Fig 3.2, 3.3, 3.4, 3.5) within 10 minutes of adding it to the recording chamber. The peak amplitude of MEPPs also showed a significant increase (Fig 3.8, 3.9), while EPP amplitude showed a slight decrease (Fig 3.6). Interestingly, resting membrane potential depolarised significantly in the presence of neostigmine (Hepes MPS; -64.07±1.2mV, control plasma; -58.34±1.5mV, dimethoate plasma; -63.19±1.4mV, pesticide plasma; -61.88±1.1mV, neostigmine; -54.73±1.5mV, mean ± SEM, $P < 0.05$, Bonferroni's, "F = 5.89") (Fig 3.7). This might have been responsible for the observed decline in EPP peak amplitude, either as a result of reduced driving force on EPP depolarization or the effects of non-quantal ACh release (Vyskocil et al., 1983; Vyskocil et al., 2009), receptor desensitization (Krnjevic and Miledi, 1958; Magleby and Pallotta, 1981; Giniatullin and Magazanik, 1998) or all these together.

In summary, dimethoate plasma (containing dimethoate and its metabolite omethoate) prolonged EPPs in a manner consistent with anticholinesterase activity reported previously in the literature. On the other hand combination of dimethoate, cyclohexanone and their metabolites (pesticide plasma) have some noticeable additive effects on synaptic transmission at the NMJ along with anticholinesterase activity. Therefore I hypothesised that effects of pesticide plasma (anticholinesterase activity along with the additive effects) may have adverse effects on synaptic transmission; hence I set out to look any for evidence for NMJ transmission failure with the pesticide plasma-treated nerve muscle preparations in the next set of experiments.

Fig. 3.2: **Intracellular endplate potential (EPP) recordings with different treatments.** A: Neostigmine (positive control) Showing substantial prolongation of synaptic potential decay, B; Control plasma (plasma from minipigs treated with saline) had no effects on EPP time course compared to Hepes MPS (trace E), C: Pesticide plasma (plasma from minipigs treated with commercially available pesticide, Dimethoate EC), D; Dimethoate plasma (plasma from minipigs treated with unformulated pesticide, i.e. pure dimethoate). Pesticide plasma (C) and dimethoate plasma (D) traces show smaller increases in EPP decay time, but not to the same extent as the positive control (neostigmine).

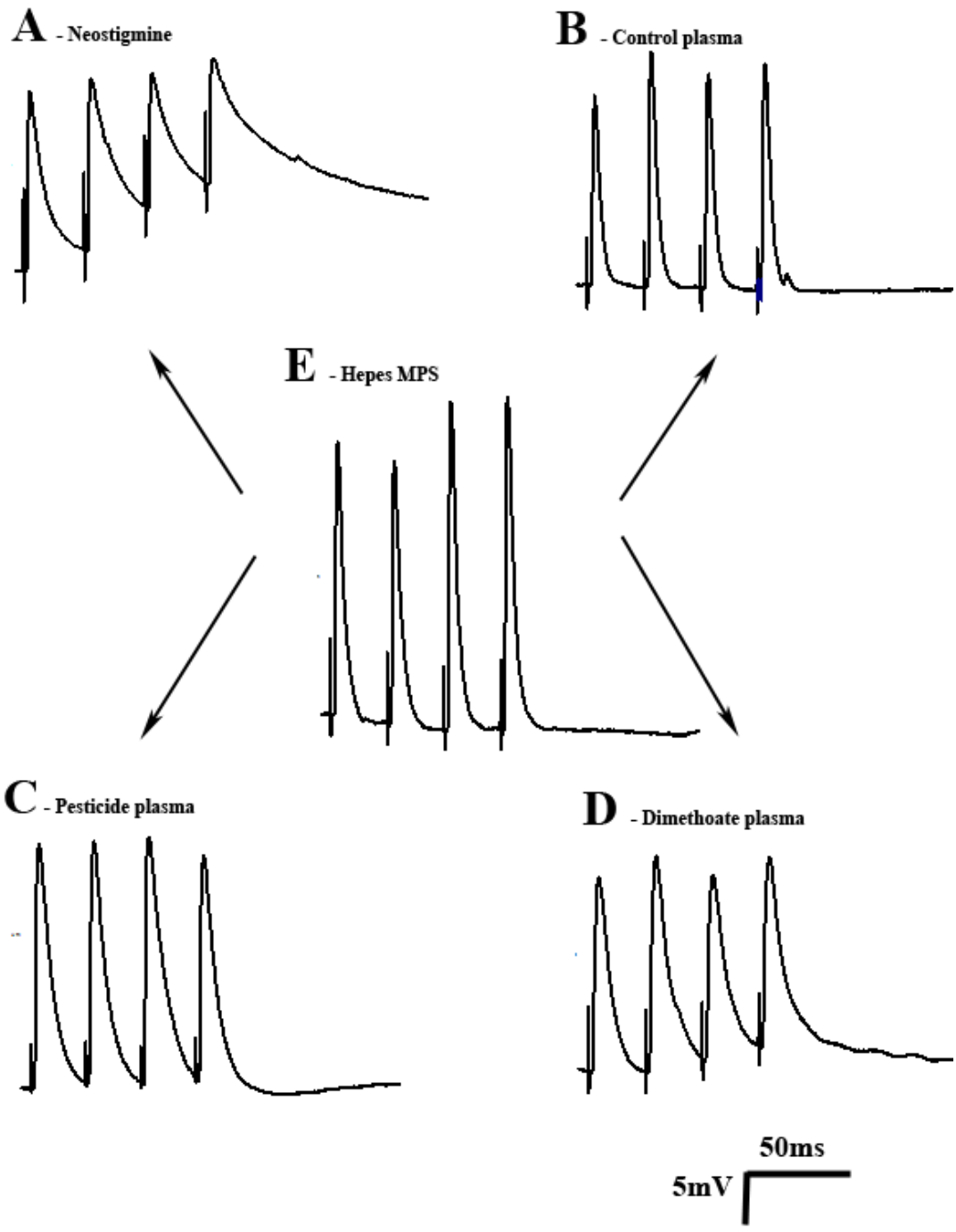

A - Neostigmine
B - Control plasma
E - Hepes MPS
C - Pesticide plasma
D - Dimethoate plasma
50ms
5mV

Fig 3.3: Half decay times (T50%) of EPPs in trains of four with different treatments. A: Half decay time of EPPs (WinWCP settings: time to decay from 90% to 50% of peak response) with different treatments. **B**: B-a: Hepes MPS Vs Neostigmine, B-b; Hepes MPS Vs Control plasma, B-c; Hepes MPS Vs Pesticide plasma, B-d; Hepes MPS Vs Dimethoate plasma. E 1 – 4 represent each subsequent EPP in T-O-F traces. Each bar represents mean ± SEM [n (No of muscles): Hepes = 14, neostigmine = 3, control plasma = 3, pesticide plasma = 5, dimethoate plasma = 3] ($p < 0.05$, **ANOVA, Bonferroni post test – treatements were compared with Hepes MPS**).

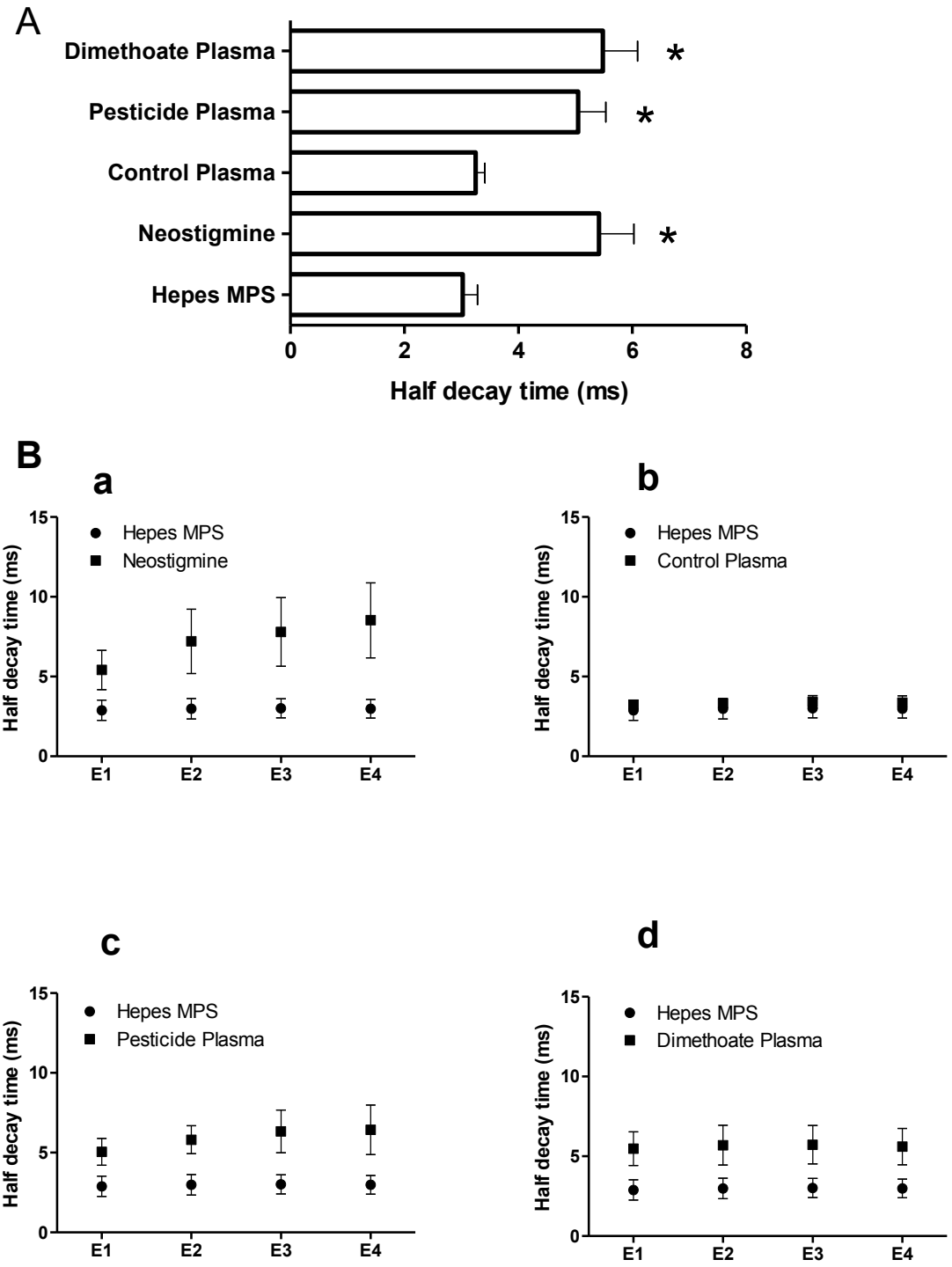
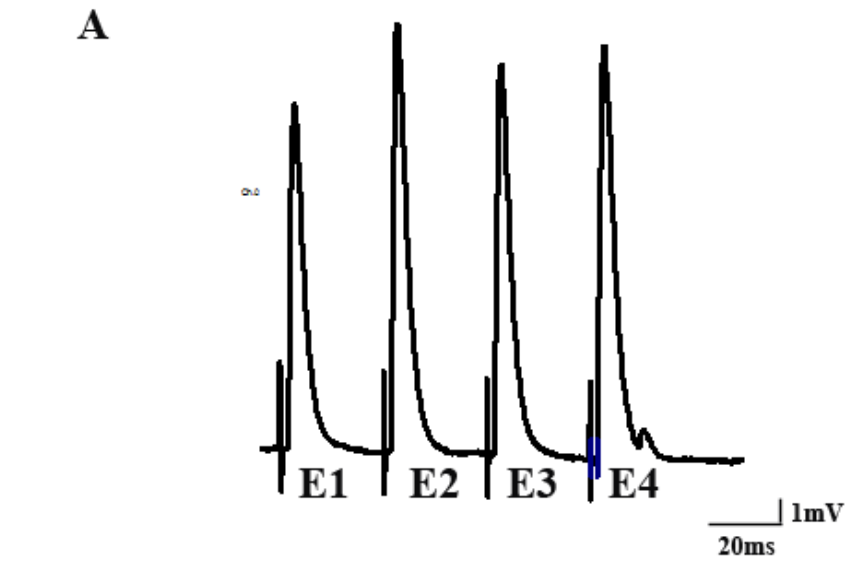

A
Dimethoate Plasma
Pesticide Plasma
Control Plasma
Neostigmine
Hepes MPS
*
*
*
0
2
4
6
8
Half decay time (ms)
B
a
Hepes MPS
Neostigmine
Half decay time (ms)
15
10
5
0
E1
E2
E3
E4
b
Hepes MPS
Control Plasma
c
Hepes MPS
Pesticide Plasma
d
Hepes MPS
Dimethoate Plasma

Fig 3.4: Half decay times facilitation of EPPs in trains of four with different treatments. A: Example train of four (E 1 – 4 represent each subsequent EPP in T-O-F traces), B: Decay time facilitation during T-O-F stimuli as per following equestion;

$$Decay\ time\ facicitation\ (T50\%) = \frac{T50\%\ of\ E4 - T50\%\ of\ E1}{T50\%\ of\ E1} * 100\%.$$

The percentage increases in half-decay time of the fourth EPP in relation to the first in subsequent (Increment of time course of fourth response compared to first response were standarzed as a percentage from the initial time course). Each bar represents mean ± SEM [n (No of muscles): Hepes = 14, neostigmine = 3, control plasma = 3, pesticide plasma = 5, dimethoate plasma = 3] ($p < 0.05$, **ANOVA, Bonferroni post test – treatements were compared with Hepes MPS**).


A
E1
E2
E3
E4
1mV
20ms

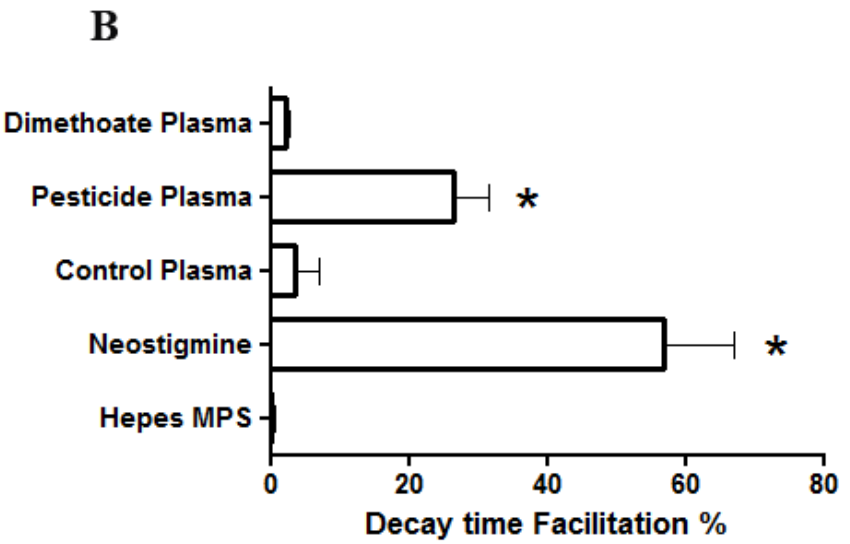

B
Dimethoate Plasma
Pesticide Plasma
Control Plasma
Neostigmine
Hepes MPS
*
*
0
20
40
60
80
Decay time Facilitation %

Fig 3.5: Time course of half decay time increase in different treatments. Half decay time of the baseline recordings were approximately 2 – 3 ms. Note that neostigmine produced prolonged decay time (≈ 6 – 7 ms) soon after treatment while dimethoate and pesticide plasma had a slow onset producing the similar effects. Data were shown from single experiment for each of the treatment (n=1). Line was fitted with linear regression to demonstrate the tendency of effects.

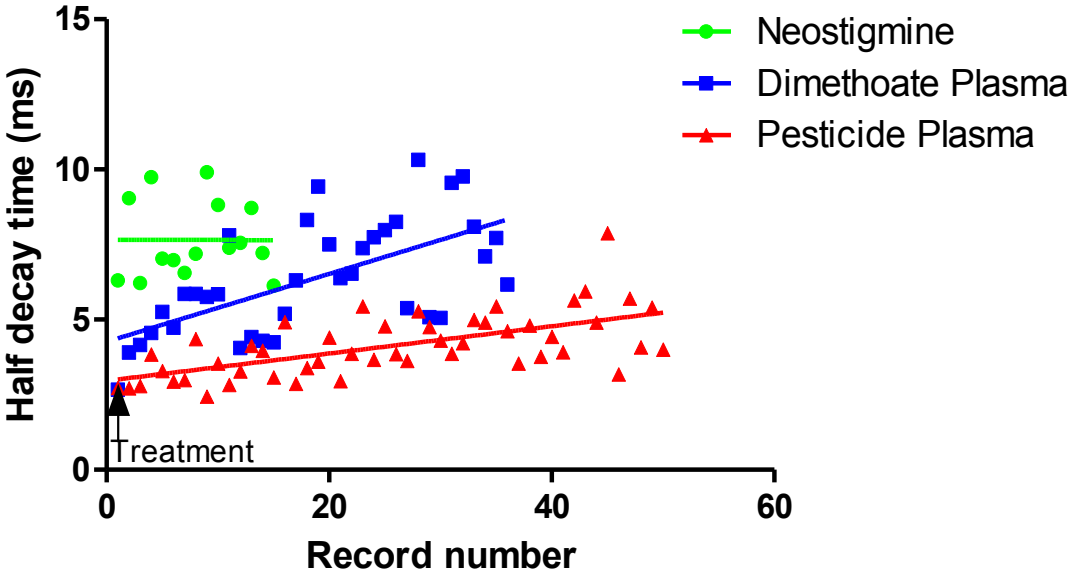

Neostigmine
Dimethoate Plasma
Pesticide Plasma
Half decay time (ms)
15
10
5
0
Treatment
0
20
40
60
Record number

Fig. 3.6: Comparison of synaptic potential rise time and peak amplitude. A; rise time of EPP-TOF responses, B; peak amplitude, of EPPs (E1) with different treatment. Note that the significant reduction in peak amplitude of EPPs in neostigmine and pesticide plasma treated samples. E 1 – 4 represent each subsequent EPP in TOF traces. Data obtained from 20min – 4hrs post treatment. Each bar represents mean ± SEM [n (No of muscles): Hepes = 14, neostigmine = 3, control plasma = 3, pesticide plasma = 5, dimethoate plasma = 3] ($p < 0.05$, **ANOVA, Bonferroni post test-treatements were compared with Hepes MPS**)

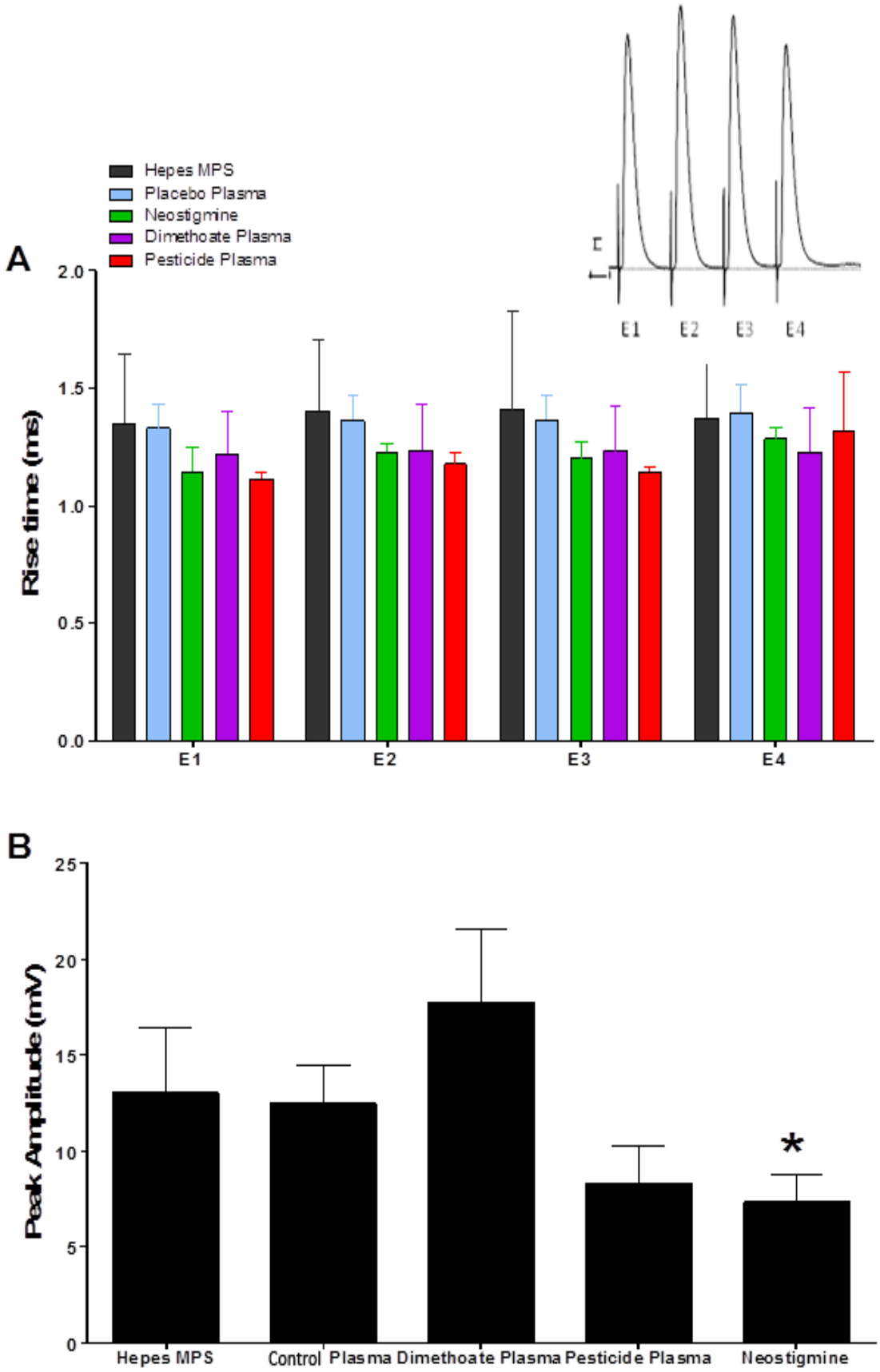

A
Hepes MPS
Placebo Plasma
Neostigmine
Dimethoate Plasma
Pesticide Plasma
Rise time (ms)
2.0
1.5
1.0
0.5
0.0
E1
E2
E3
E4
B
Peak Amplitude (mV)
25
20
15
10
5
0
*
Hepes MPS
Control Plasma
Dimethoate Plasma
Pesticide Plasma
Neostigmine

Fig 3.7: Comparison of change in resting membrane potential in response to drug treatments. Data was obtained during the period of 20min – 4hrs post treatment. Only neostigmine treatment produced a significant depolarization of the resting membrane potential. Other treatments produced no significent change, in comparison to Hepes MPS. Data reprecent a cumulative value from all the experiments. Each bar represents mean value ± SEM (fibres) [n (No of muscles): Hepes = 14, neostigmine = 3, control plasma = 3, pesticide plasma = 5, dimethoate plasma = 3] ($p < 0.05$, **ANOVA, Bonferroni post test - treatements were compared with Hepes MPS**)

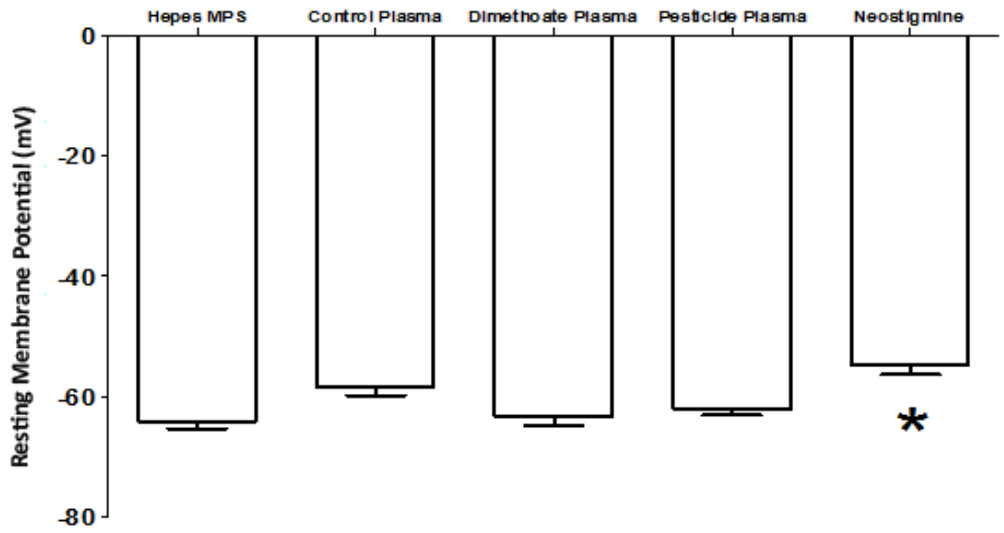

Hepes MPS
Control Plasma
Dimethoate Plasma
Pesticide Plasma
Neostigmine
Resting Membrane Potential (mV)
0
-20
-40
-60
-80
*

Fig 3.8: Intracellular MEPPs recordings with different treatments. A-a: Hepes MPS, B-b: neostigmine, C-c: pesticide plasma, D-d: dimethoate plasma. Note the pesticide plasma treated samples displayed an increase MEPP frequency (C). A – D traces represent a slower time base and show the distribution of MEPP, while a – d traces represent a fast time base and demonstrate characteristics of individual MEPPs.

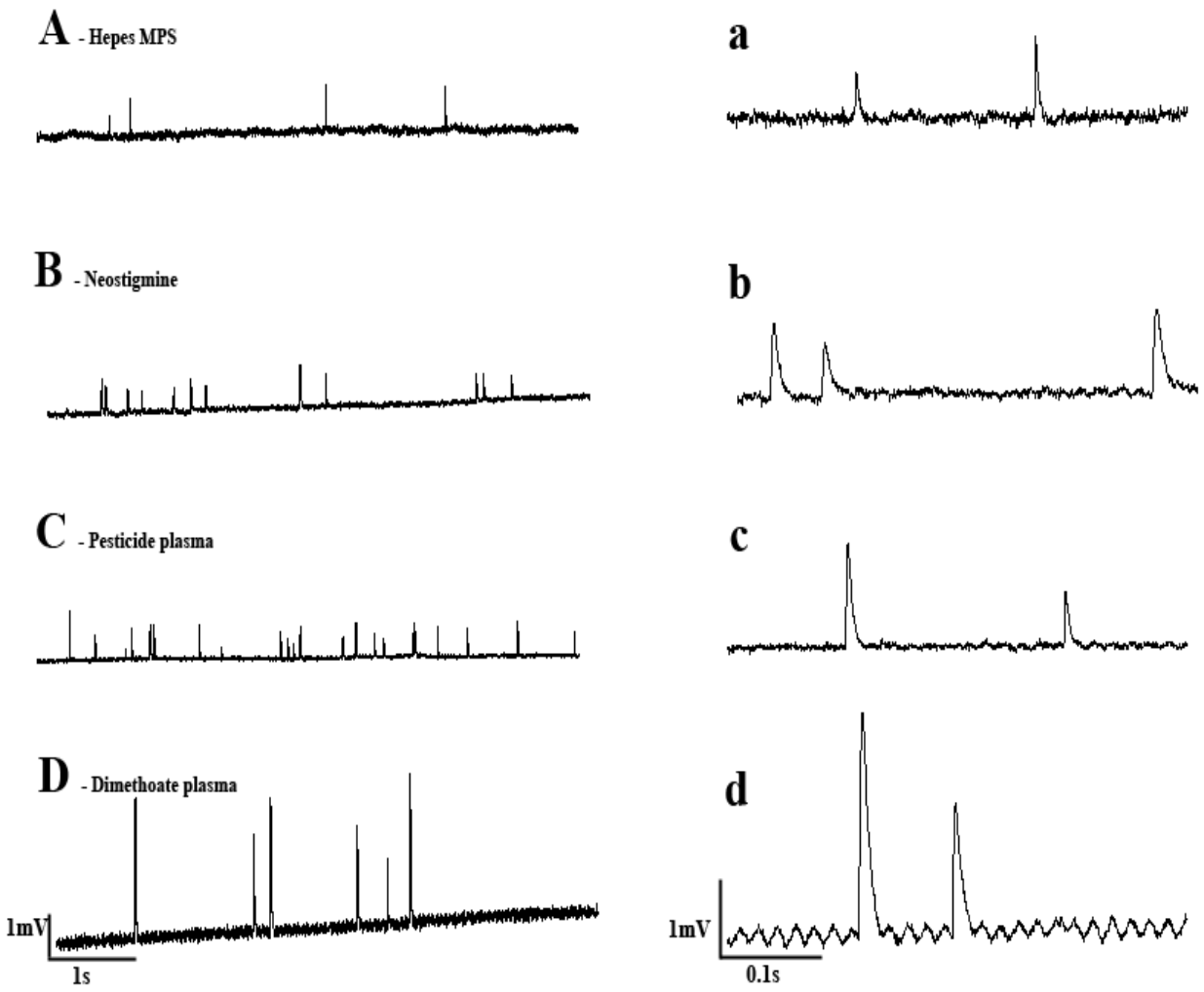
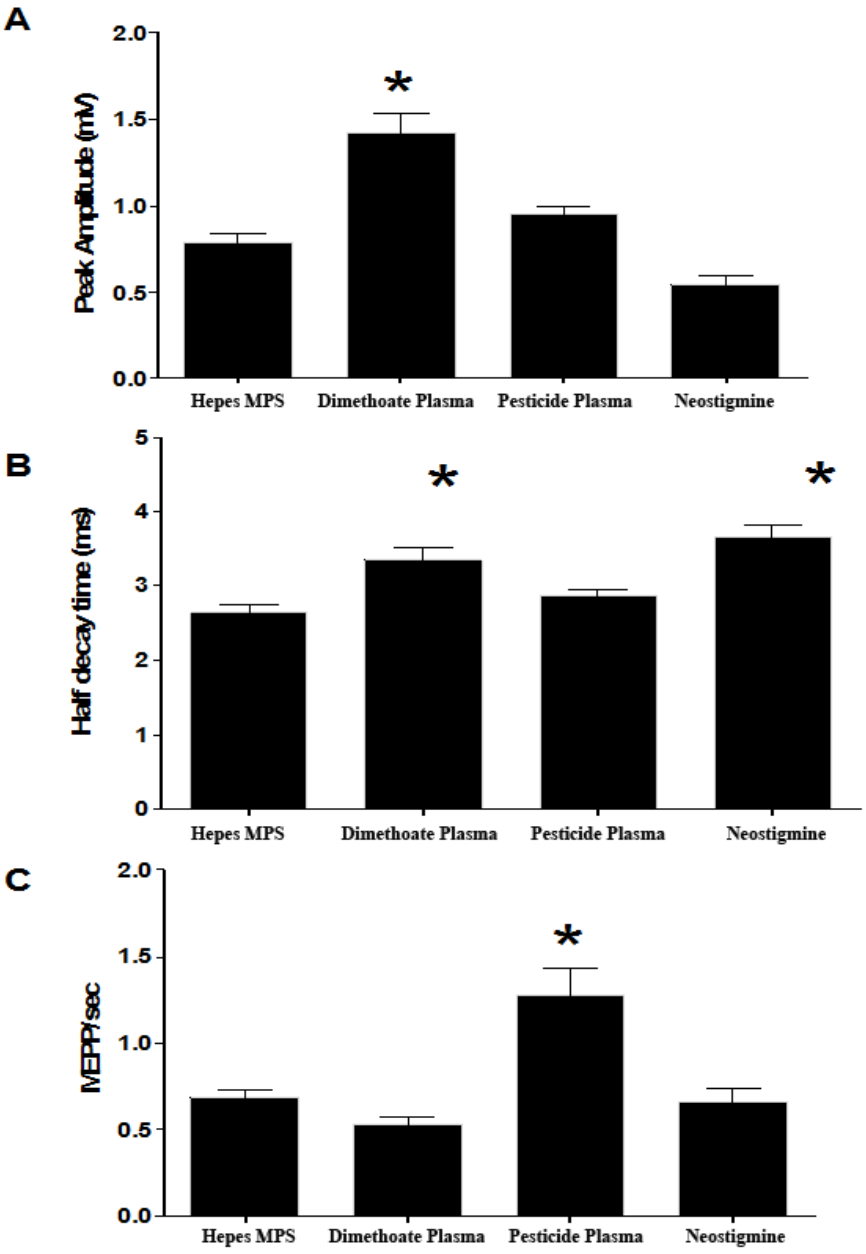

A - Hepes MPS
a
B - Neostigmine
b
C - Pesticide plasma
c
D - Dimethoate plasma
d
1mV
1s
1mV
0.1s

Fig 3.9: Comparison of MEPPs parameters with different plasma treatment: A; Peak amplitude, B; Half decay time, C; MEPP frequency. Dimethoate plasma MEPPs showed a significant increase in both peak amplitude and half decay time comapared to Hepes MPS, while pesticide plasma MEPPs showed significant increment in MEPPs frequency compared to Hepes MPS. Neostigmine treated samples MEPPs also showed a significant increase in half decay time compared to Hepes MPS. Data obtained from 20min – 4hrs from treatment, each represent mean frequency ± SEM [n (No of muscles): Hepes = 14, neostigmine = 3, pesticide plasma = 5, dimethoate plasma = 3] ($p < 0.05$, **ANOVA, Bonferroni post test - treatements were compared with Hepes MPS**)


A
Peak Amplitude (mV)
2.0
1.5
1.0
0.5
0.0
*
Hepes MPS
Dimethoate Plasma
Pesticide Plasma
Neostigmine
B
Half decay time (ms)
5
4
3
2
1
0
*
*
Hepes MPS
Dimethoate Plasma
Pesticide Plasma
Neostigmine
C
MEPP/sec
2.0
1.5
1.0
0.5
0.0
*
Hepes MPS
Dimethoate Plasma
Pesticide Plasma
Neostigmine

3.3.2. Prolonged exposure to pesticide plasma produces neuromuscular block

The hallmark of IMS is muscle paralysis. Therefore, I asked next whether minipig plasma would cause a neuromuscular transmission block. Plausible evidence for NMJ transmission block was observed with pesticide plasma treated preparations, as there were no detected EPP responses upon stimulation after 2- 4hrs. The incidence of evoked transmission failure emerged shortly after EPPs began to show prolongation of their decay time, which was also 2 – 4 hrs post treatment. Interestingly, these effects were reversible, as EPPs recovered after the 3 – 4 cycles of washing with Hepes MPS (Fig 3.10). Even though EPPs could not be evoked in these muscle fibres, they still produced MEPPs, suggesting that spontaneous transmission was unaffected. Nevertheless, this obstruction of evoked transmission was only observed with formulated pesticide plasma treated samples, while other treatments produced no such effects (Table 3.1).

In summary, the data suggested the complex nature of the effects of pesticide plasma on neuromuscular transmission consistent with data from pig studies in vivo. My preliminary findings are consistent with the pig data suggest perhaps that the anticholinesterases activity of dimethoate alone is not sufficient to explain IMS. Data from the pig study (Eddleston et al., 2012) implicated roles for pesticide solvent (cyclohexanone) and the metabolites of both dimethoate and cyclohexanone, namely omethoate and cyclohexanol. I therefore turned my attention next the *in-vitro* effects of these components.

Fig 3.10: Traces showing evoked synaptic transmission failure following addition of pesticide plasma to the bathing medium. Traces: A; Hepes MPS, B; pesticide plasma showing prolonged EPP decay, C; pesticide plasma showing complete block of neuromuscular transmission, (Note that there is only stimulus artefact along with MEPPs are present) D; Hepes MPS (after pesticide plasma was washed off, showing evidence of prolongation of EPP suggesting residue anticholinesterase action). Blue arrows indicate the MEPPs.

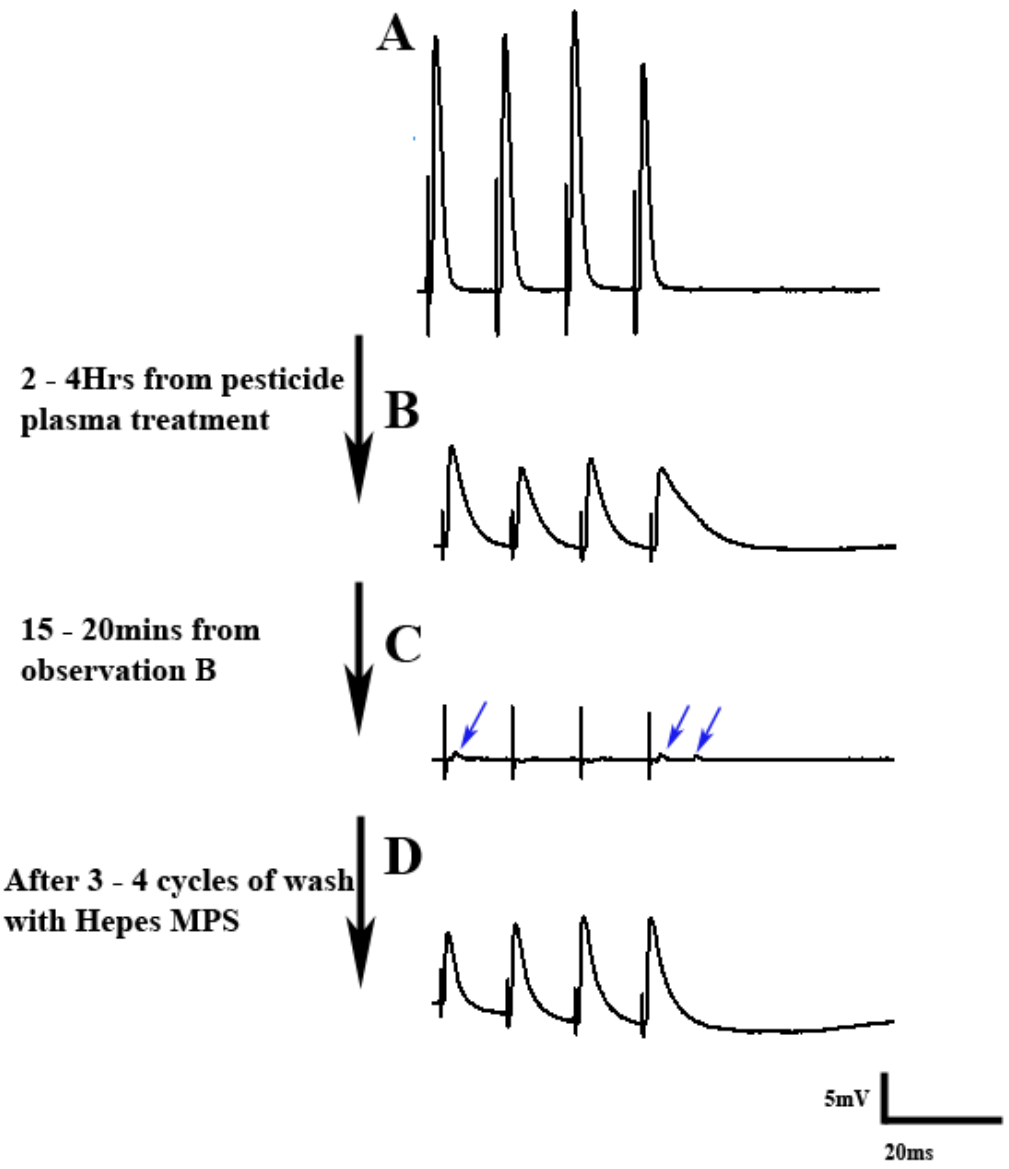

A
2 - 4Hrs from pesticide plasma treatment
B
15 - 20mins from observation B
C
After 3 - 4 cycles of wash with Hepes MPS
D
5mV
20ms

Table 3.1: Summary data of electrophysiological evaluation of synaptic transmission at NMJ in minipig plasma treated preparations. Note that EPPS remained in only one experiment out of 6 for the muscles treated with pesticide plasma

	Mean membrane potential (Em) during the course of experiment	Frequency of MEPPs throughout the experiment	No of experiments in with EPPs were evoked by nerve stimulation at the beginning of procedure	No of experiments with EPPs at the end of procedure
Neostigmine	**-54.73±1.5mV**	**0.66±0.4434s^{-1}**	**4/4**	**4/4**
Control Plasma	**-58.34±1.5mV**	**0.68±0.34s^{-1}**	**3/3**	**3/3**
Dimethoate Plasma	**-63.19±1.4mV**	**0.53±0.2734s^{-1}**	**3/3**	**3/3**
Pesticide Plasma	**-61.88±1.1mV**	**1.27±1.2034s^{-1}**	**6/6**	**1/6**

3.3.3 Omethoate and cyclohexanol in combination synergistically prolong synaptic transmission

If the hypothesis that the ingredients of pesticide and/or their metabolites required for the effects of pesticide plasma is correct then we would predict that the parent compounds in pesticide and their metabolic breakdown products would have additive or synergistic effects on NMJ synaptic transmission.

I therefore test the effects of dimethoate, omethoate, cyclohexanone and cyclohexanol together referred to as (DOCC) singly and in various combinations.

First, it was necessary to examine how individual components of the pesticide and their metabolites affect synaptic transmission. I measured rise time, amplitude and decay time of synaptic potentials in this experiment. Secondly, I sought to test the treatment potency of each individual compound on neuromuscular transmission. I used prolongation of EPP decay time as an index of anticholinesterase activity. Thirdly, I tested whether it was possible to reproduce neuromuscular transmission block using combinations of the DOCC components. The objective of this set of experiments was to identify whether combinations of these molecules might be responsible for the failure of neuromuscular transmission observed in human pesticide-poisoned patients with IMS and the decline of mechanomyographic responses observed in the intensive care minipig model of OP toxicity (Eddleston et al., 2012).

Fig 3.11 shows sample traces of EPPs recorded using different drug treatments. Traces were further analysed for their post synaptic characteristics: that is, their rise time, peak amplitude and half decay time.

Inspection of single EPPs in different treatments suggested prolongation of synaptic potentials with dimethoate, omethoate, cyclohexanol and their combinations but not with cyclohexanone (fig 3.11). Quantitative analysis of EPPs at 1Hz confirmed the significant increment of half decay time with omethoate compared to Hepes MPS (fig 3.12A, table 3.2). I also found that cyclohexanol prolonged synaptic transmission, showing a significant increase in decay time (fig 3.12A, table 3.2). Interestingly, an additive effect on half decay time was observed in preparations when omethoate and cyclohexanol were added to the bathing medium (fig 3.11, 3.12A, table 3.2). Surprisingly, peak amplitudes of EPPs (fig 3.12B, table 3.2) showed a reduction, in neostigmine, dimethoate, cyclohexanol and combination of omethoate and DOCC treated preparations.

The effect of omethoate on cholinesterase activity was measured in a biochemical assay and the results are shown in Figure 3.13. (These measurements were carried out by Dr. V. Patel and Dr. L.G. Sultatos at New Jersey Medical School-Rutgers University, Newark, USA). It is evident that 100µM omethoate completely inhibits AChE activity. Cyclohexanol (5mM) also inhibits AChE activity but only by about 25 – 30% Thus, when considering the mechanisms for additive effects of omethoate and cyclohexanol on half decay time of EPPs, these data together suggest that the effects of cyclohexanol in combination with omethoate cannot be explained simply by additional inhibition of AChE, since this enzyme is completely inhibited by omethoate alone at the concentrations present in poisoned plasma and in my in vitro experiments. Cyclohexanol must therefore prolong EPPs by some other mechanism in addition to its mild anti-AChE effects.

More pronounced effects on EPP decay were observed with low concentrations of omethoate (EC 50; 2.75µM), compared with dimethoate (EC 50: 1.4mM) (fig 3.14). Both cyclohexanone and cyclohexanol were also tested for prolongation of synaptic potentials using the same criteria of increase half-decay time as a measure. Ethanol was tested for comparison with since it is also a substrate for alcohol dehydrogenase and it is already known to affect the NMJ transmission (Searl and Silinsky, 2010b, a). The data show that both cyclohexanol and ethanol potentiate EPP decay at higher concentration of the drug (mM), while cyclohexanone has no discernible effects on EPP decay time even at higher concentrations (mM) (fig 3.15).

Together therefore, these results indicate that the metabolic breakdown products omethoate and cyclohexanol have more potent effects on decay kinetics of neuromuscular transmission compared to their parent components, dimethoate and cyclohexanone.

Fig 3.11: Comparison of EPPs with different combination of pesticide components. A; Hepes MPS, B; neostigmine (10µM), C; dimethoate (1mM), D; omethoate (100µM), E; cyclohexanone (1mM), F; cyclohexanol (5mM), G; Omethoate and cyclohexanol, H; dimethoate, omethoate, cyclohexanone, cyclohexanol (DOCC). Note that decay time of EPP is longest when all four components of pesticide are combined together. Treatment concentrations for were adopted from Eddleston el al 2012, based on plasma concentrations in the Göttingen minipig model of OP-pesticide poisoning

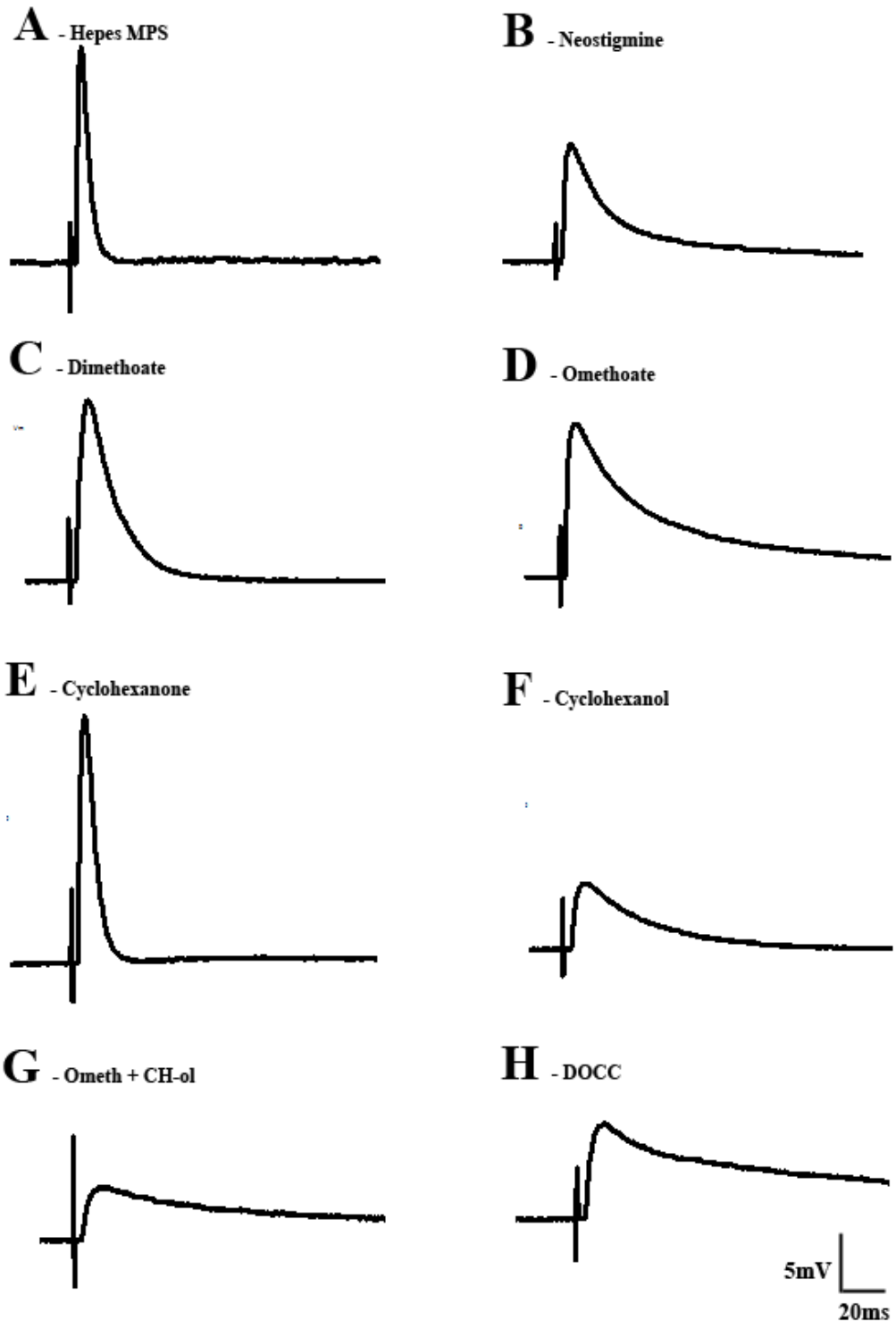
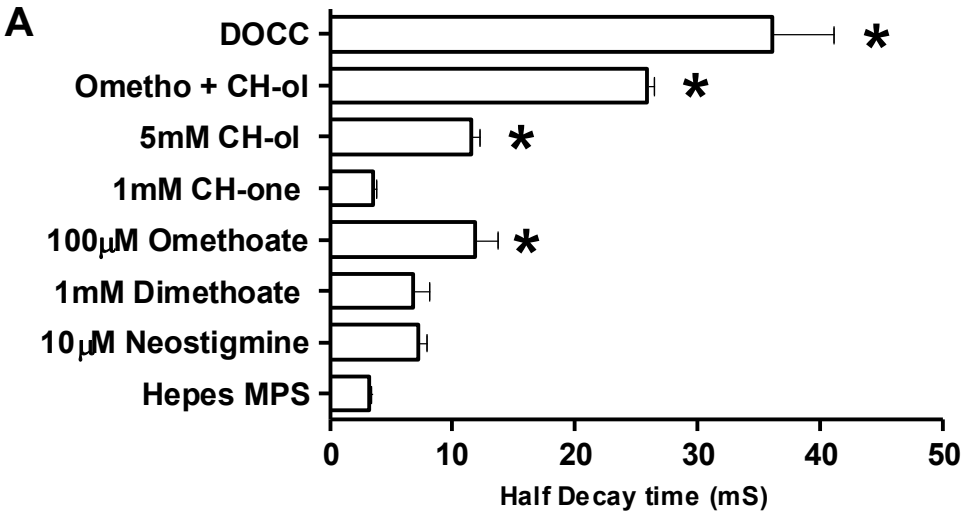

A - Hepes MPS
B - Neostigmine
C - Dimethoate
D - Omethoate
E - Cyclohexanone
F - Cyclohexanol
G - Ometh + CH-ol
H - DOCC
5mV
20ms

Table 3.2: Summary of statistical analysis of post synaptic parameters with pesticide ingredients. Statistical test; oneway analysis of variance. Post test; Bonferroni multiple comparison test. (CH-one, CH-ol and DOCC represent, cyclohexanone cyclohexanol and dimethoate, omethoate, cyclohexanone and cyclohexanol respectively). N = 3 muscles per each treatment groups, n = 6 – 10 fibres per muscle.

Half Decay time (mS)					
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Hepes MPS vs 1mM Dimethoate	-3.573	2.379	No	ns	-7.848 to 0.7024
Hepes MPS vs 100µM Omethoate	-8.634	5.116	Yes	***	-13.44 to -3.829
Hepes MPS vs 1mM CH-one	-0.2901	0.1719	No	ns	-5.095 to 4.515
Hepes MPS vs 5mM CH-ol	-8.333	4.937	Yes	***	-13.14 to -3.528
Hepes MPS vs Ometho + CH-ol	-22.63	13.41	Yes	***	-27.44 to -17.83
Hepes MPS vs DOCC	-32.8	19.43	Yes	***	-37.61 to -28.00
Peak Amplitude (mV)					
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Hepes MPS vs 1mM Dimethoate	8.791	4.599	Yes	***	3.349 to 14.23
Hepes MPS vs 100mM Omethoate	4.426	2.06	No	ns	-1.691 to 10.54
Hepes MPS vs 1mM CH-one	0.7922	0.3687	No	ns	-5.324 to 6.909
Hepes MPS vs 5mM CH-ol	11.8	5.492	Yes	***	5.683 to 17.92
Hepes MPS vs Ometho + CH-ol	14.27	6.643	Yes	***	8.155 to 20.39
Hepes MPS vs DOCC	12.4	5.773	Yes	***	6.286 to 18.52

Fig 3.12: Comparison of EPP parameters with different combinations of the pesticide components. A: half decay time B; peak amplitude. Prolongation of decay time is largest when either two metabolites or all four ingredients of pesticides are combined together. Note the additive effects of omethoate and cyclohexanol on decay time when combined together. Each bar represent mean ± SEM (n = 3 muscles per each treatment group) ($p < 0.05$, **ANOVA, Bonferroni post test- treatements were compared with Hepes MPS**).


A
DOCC
Ometho + CH-ol
5mM CH-ol
1mM CH-one
100μM Omethoate
1mM Dimethoate
10μM Neostigmine
Hepes MPS
0
10
20
30
40
50
Half Decay time (mS)

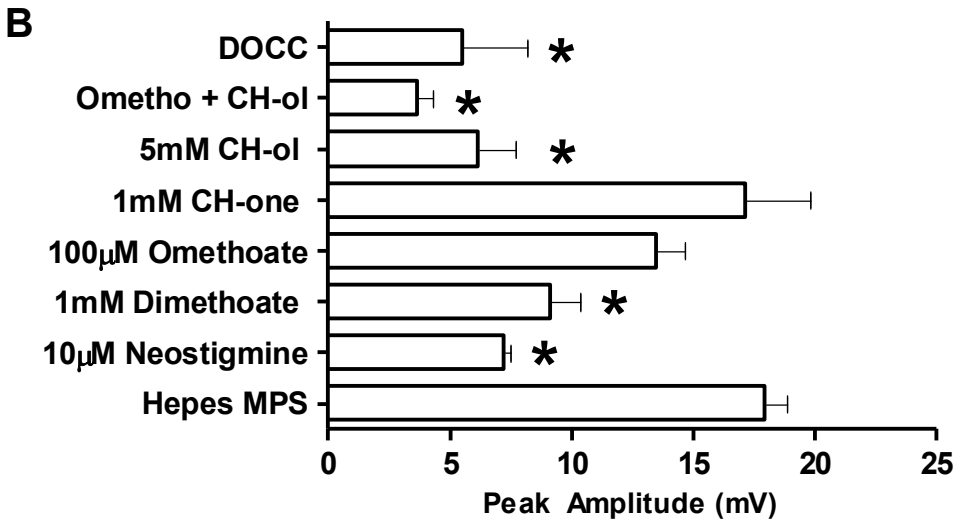

B
DOCC
Ometho + CH-ol
5mM CH-ol
1mM CH-one
100μM Omethoate
1mM Dimethoate
10μM Neostigmine
Hepes MPS
0
5
10
15
20
25
Peak Amplitude (mV)

Fig 3.13: AChE activity after 30 minutes incubation of the treatment at room temperature. Each data point represent mean ± SEM (n = 6 muscles per each treatment group) ($p < 0.05$, **ANOVA, Dunnett's post test**). Experiments of AChE activity was carried out by Dr. V. Patel and Dr. L.G. Sultatos at New Jersey Medical School-Rutgers University, Newark, United States of America.

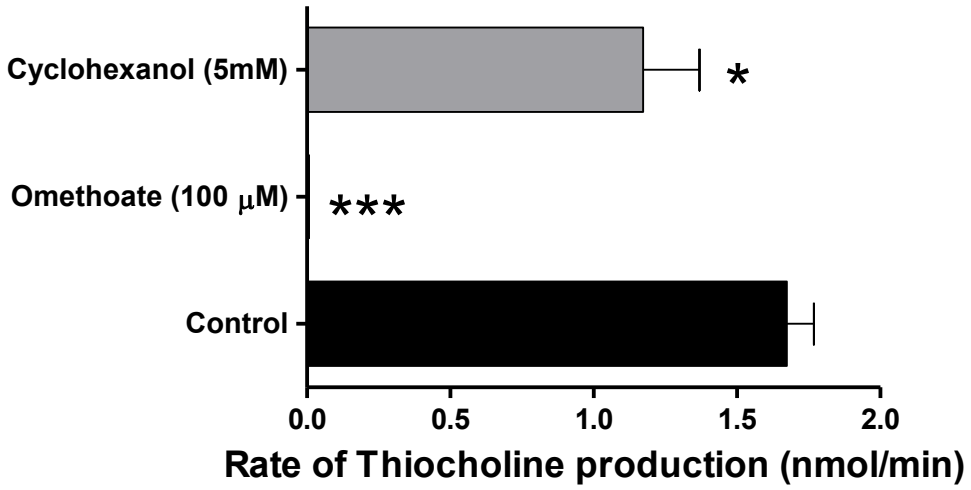
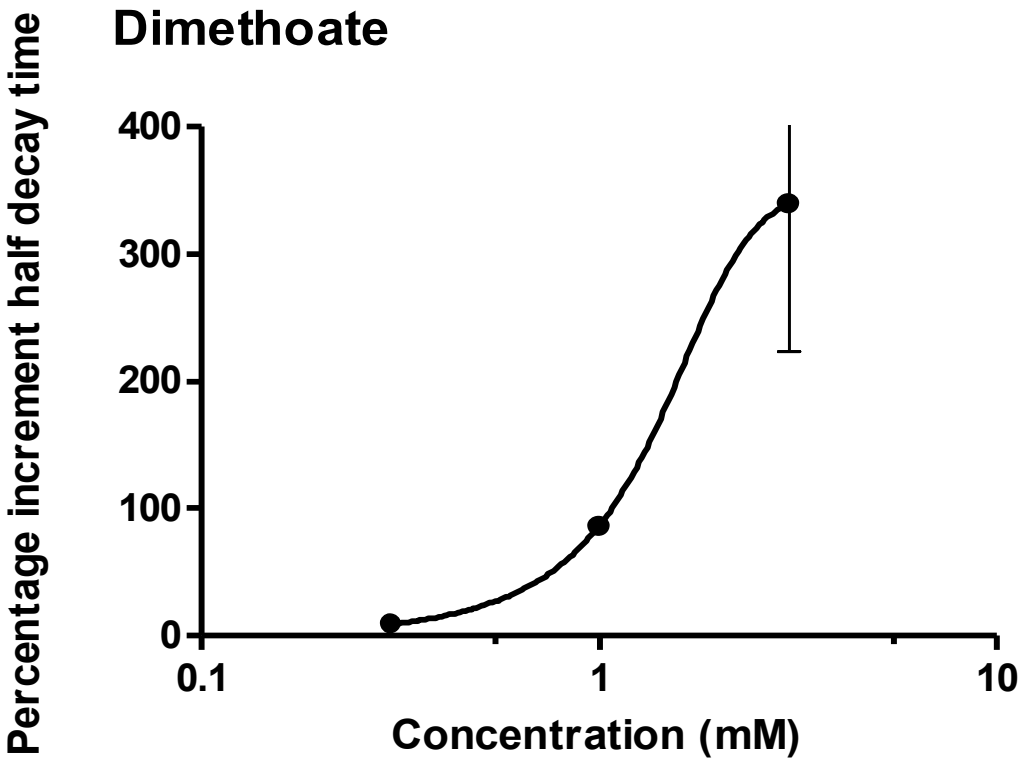

Cyclohexanol (5mM)
Omethoate (100 μM)
Control
*
***
0.0
0.5
1.0
1.5
2.0
Rate of Thiocholine production (nmol/min)

Fig 3.14: Change of half decay time with drug concentration. Effective concentration **(**Ec) 50 value for dimethoate and omethoate are 1.4mM and 2.75μM respectively. Note that the low Ec 50 value of omethoate compared to dimethoate. Each data point represent the mean half decay time ± SEM (n = 3 muscles per each treatment group).


Dimethoate
Percentage increment half decay time
400
300
200
100
0
0.1
1
10
Concentration (mM)

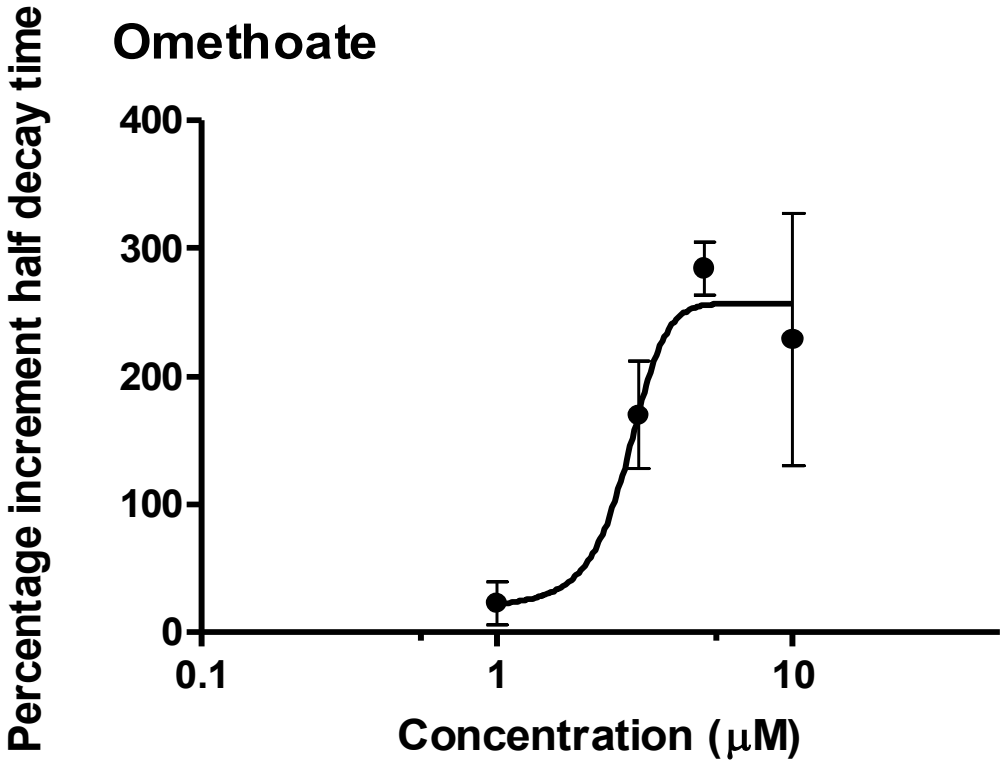
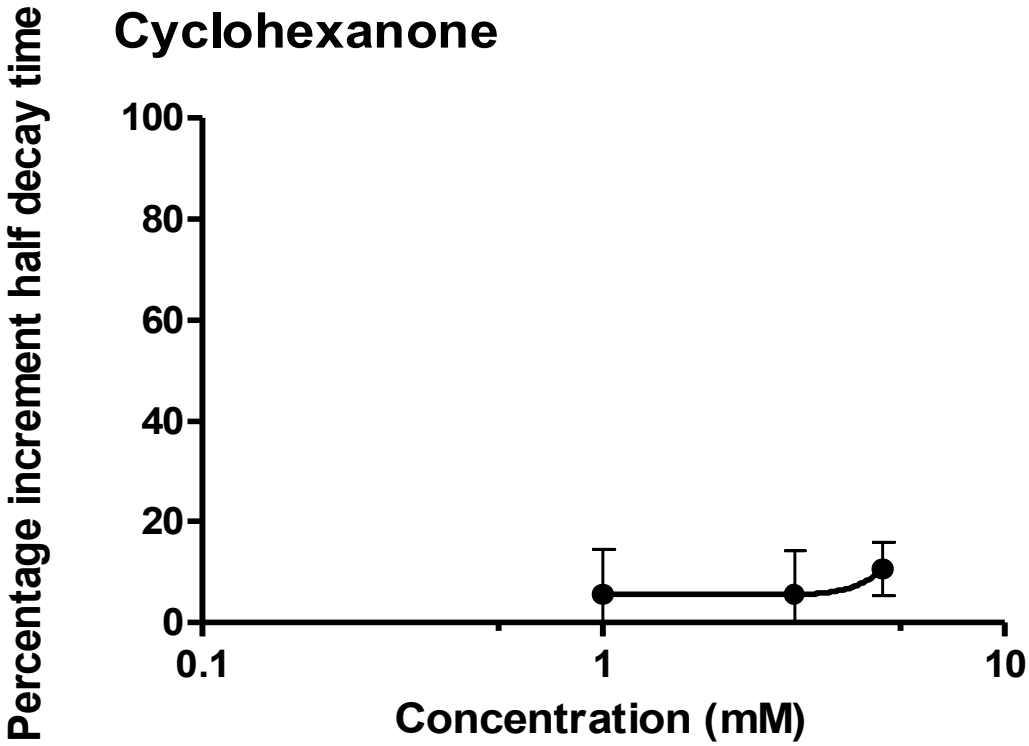
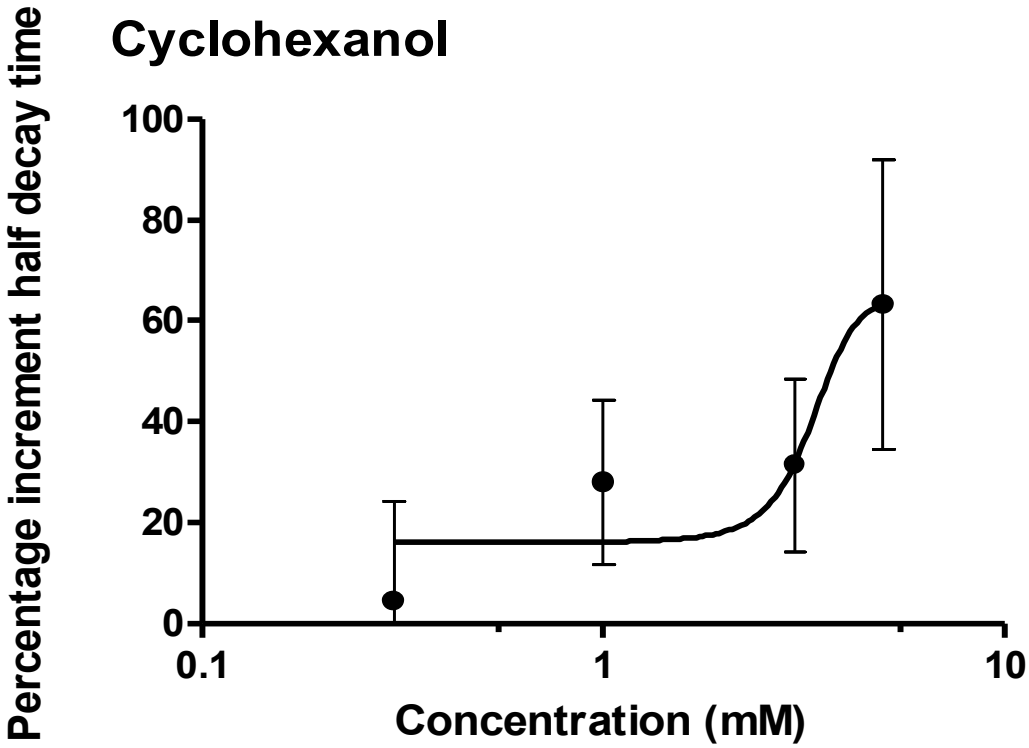

Omethoate
Percentage increment half decay time
400
300
200
100
0
0.1
1
10
Concentration (μM)

Fig. 3.15: Change of half decay time with drug concentration. Cyclohexanol had a threshold (maximum) effect of 3mM and at 5mM produced about 60% increase in half decay time of EPP; where as the effects of cyclohexanone was barely detectable at 5mM (see also fig 3.12A). Cyclohexanol have had detectable effects on decay time of the synaptic potential, while cyclohexanone produce no effects. Each bar represent the mean half decay time ± SEM (n = 3 muscles per each treatment group).


Cyclohexanone
Percentage increment half decay time
100
80
60
40
20
0
0.1
1
10
Concentration (mM)


Cyclohexanol
Percentage increment half decay time
100
80
60
40
20
0
0.1
1
10
Concentration (mM)

3.3.4 Patterned repetitive stimulation at high frequencies produce short term synaptic depression with different combinations of pesticide ingredients and metabolites

I next examined for evidence of synaptic depression on NMJ transmission failure using high frequency (10Hz -50Hz) trains of stimulation with varying intervals between trains (fig 3.16). Example traces of trains (10) of stimuli (50Hz) from Hepes MPS recording are shown in figure 3.17. Qualitative evaluation was carried out for responsiveness of the muscle fibre for stimulation and run down of EPP trains during high frequency stimulation. μ-CTX pretreated preparations in MPS do not produce muscle action potentials or mechanical twitch upon stimulation. However, some responses with omethoate and its combinations were more complex, indirectly producing counteraction of μ-CTX effects, resulting in a muscle action potentials and mechanical twitch upon nerve stimulation. In baseline Hepes MPS, the threshold nerve stimulation intensity required to reliably produce a train EPPs was about 1 – 2 V (pulse width 0.1ms). Under these conditions, trains of stimuli delivered at frequencies up to 100Hz produced EPPs in response to every stimulus in the train (Figure 3.16, 3.17). However, in the presence of cyclohexanol, a higher stimulus intensity was required (> 10V) to robustly produce trains of EPPs. I frequently observed intermittent or complete absence of EPPs during trains of stimuli at 10 - 50Hz at stimulus intensities that were sufficient to generate EPP trains at high frequency in normal MPS (Fig 3.18). The effect in cyclohexanol was overcome by increasing the stimulus intensity. I did not investigate this further (but see Chapter 4) and for the remaining experiments I simply ensured that stimulus intensity was adequate to evoke consistent response in preparations treated with cyclohexanol.

Sample traces of 50Hz trains of stimuli with treatment are summarised in figure 3.19. Cumulative depolarization leading to facilitation of the EPP responses, along with a base line shift were observed in both omethoate and cyclohexanol treated preparations. Sustained depolarization upon which EPPs of decreasing amplitude were superimposed along with the prolonged decay time of the EPP are likely to be responsible for the observed summation of the responses. The baseline shift was also probably a consequence of accumulation of ACh in the synaptic cleft. However, the apparent initial temporal summation along with the slight rundown of the subsequent EPP (amplitude) responses was more noticeable in combinations of either metabolic breakdown products (omethoate and cyclohexanol) or in preparations treated with all four compounds tested. The synergestic effects of omethoate and cyclohexanol combination was observed with analysis of half decay time of the last EPP response of the 50Hz trains of stimuli (Fig 3.19 H).

I also observed possible evidence for ACh receptor desensitization during high frequency trains of stimuli in combinations of omethoate and cyclohexanol, and in DOCC-treated preparations, as EPP amplitudes appeared to become disproportionately reduced in amplitude with the prolongation of the train (Fig 3.20).

Most remarkably, profound effects of omethoate and cyclohexanol were observed in trains of qualitative analysis of response in repetitive stimulation (50Hz) (fig 3.21). In Hepes MPS, multiple cycles of high frequency bursts of stimuli produced short term synaptic depression within each cycle. However, there was no apparent fatigue, since the first EPP in each subsequent cycle produced similar responses, demonstrating fairly quick recovery of synaptic transmission. Possible evidence for desensitisation was also minimal as; a) the peak amplitude of the EPPs was comparatively stable after initial short term depression in subsequent stimulus trains, b) decay time of the EPPs were also relatively constant in subsequent stimulus trains. However when DOCC was added to the recording chamber, it showed a baseline shift along with the temporal summation and prolonged decay time of the first EPPs response. Interestingly, subsequent cycles showed reduction in temporal summation along with the decline in prolonged decay compared to first response. These observations suggest depression of synaptic transmission with DOCC with repetitive stimulation at higher stimulation frequencies. Reduction in prolonged decay with subsequent cycles also suggests possible desensitization of the junctional ACh receptors. Omethoate in combination with cyclohexanol also produced similar effects, but the magnitude of the response reduction with subsequent cycles was largest when DOCC was present.

Collectively, these data suggest that the parent components of pesticide ingredients have smaller effects on synaptic transmission at the neuromuscular junction than their metabolites. However, the effects were more pronounced when all four components were combined. Therefore, next I set out to examine, what are the consequences of these alterations on functional neuromuscular transmission, using the force of muscle contraction as a measure.

Fig 3.16: Sample traces of EPPs recorded in different frequencies of stimulation. Traces obtained in Hepes MPS. A; 1Hz, B; 10Hz, C; 20Hz. The EPPs in B show synaptic depression with repetitive stimulation. It is also evident that short term synaptic depression becomes quicker with higher stimulating frequencies, as it took 1s to produce similar amount of depression at 10HZ (Trace B) while at 20Hz (trace C) it only required 500ms. Trace C also shows evidence of slight facilitation of the first three EPPs.Vertical scale represent all three traces.

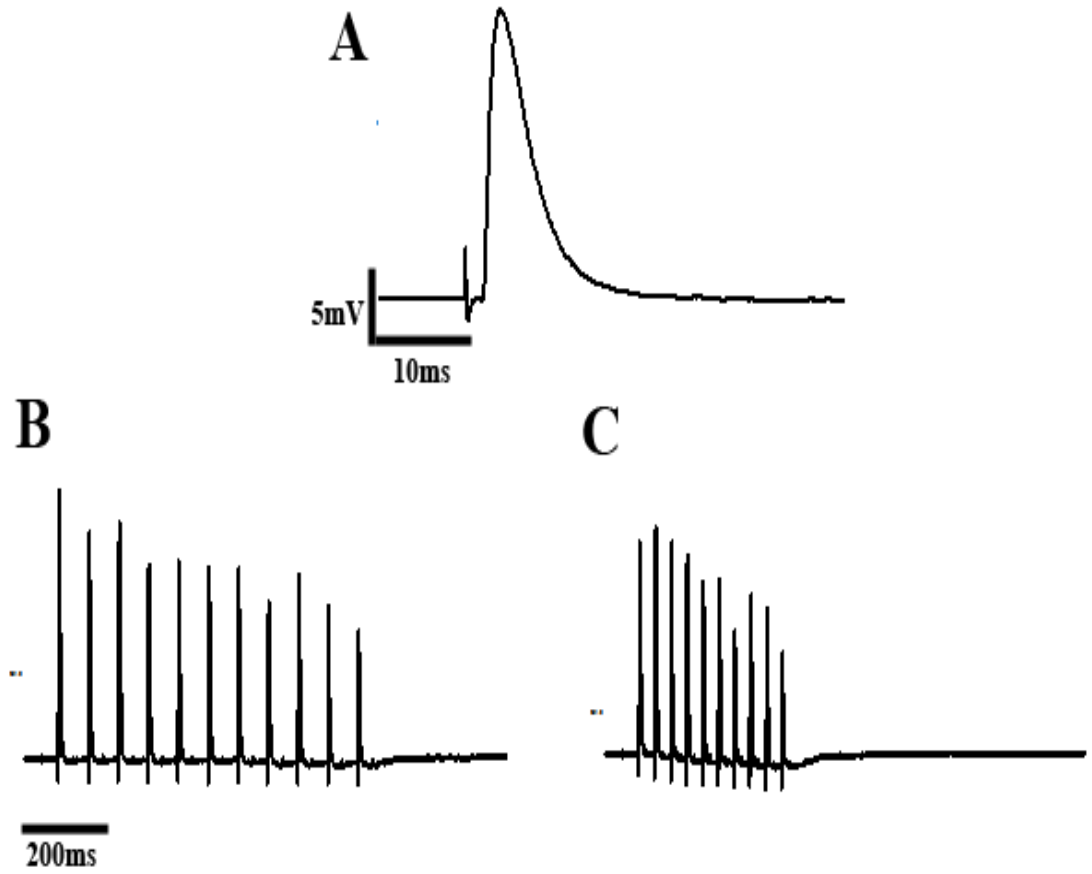

A
5mV
10ms
B
C
200ms

Fig 3.17: Sample traces from 50Hz trains of stimuli with different intervals between subsequent trains. Traces obtained in Hepes MPS. A: three successive trains of stimuli (10 stimuli per train) with an interval of 5 s between each train, B; three successive trains of stimuli (10 stimuli per train) with an interval of 2.5 s between each train. In both A and B, EPP traces shows no evidence of fatigue (reduction in EPP amplitude) in successive cycles, suggesting shorter cycle length does not alter synaptic transmission in Hepes MPS.

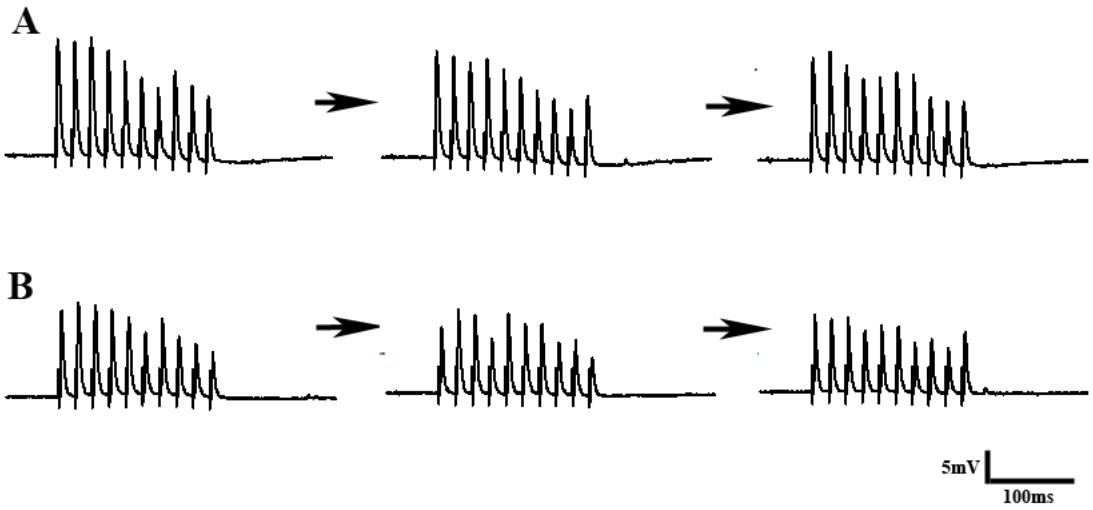

A
B
5mV
100ms

Fig 3.18: Comparison of different responses observed with cyclohexanol (5mM) at stimulating frequency of 20Hz. A; Responsive fibre producing EPP at each stimuli. Note the summation and prolonged decay time of EPPs B; Intermittent failures during a train of stimuli C; unresponsive fibre produce no EPPs in response to stimulation.

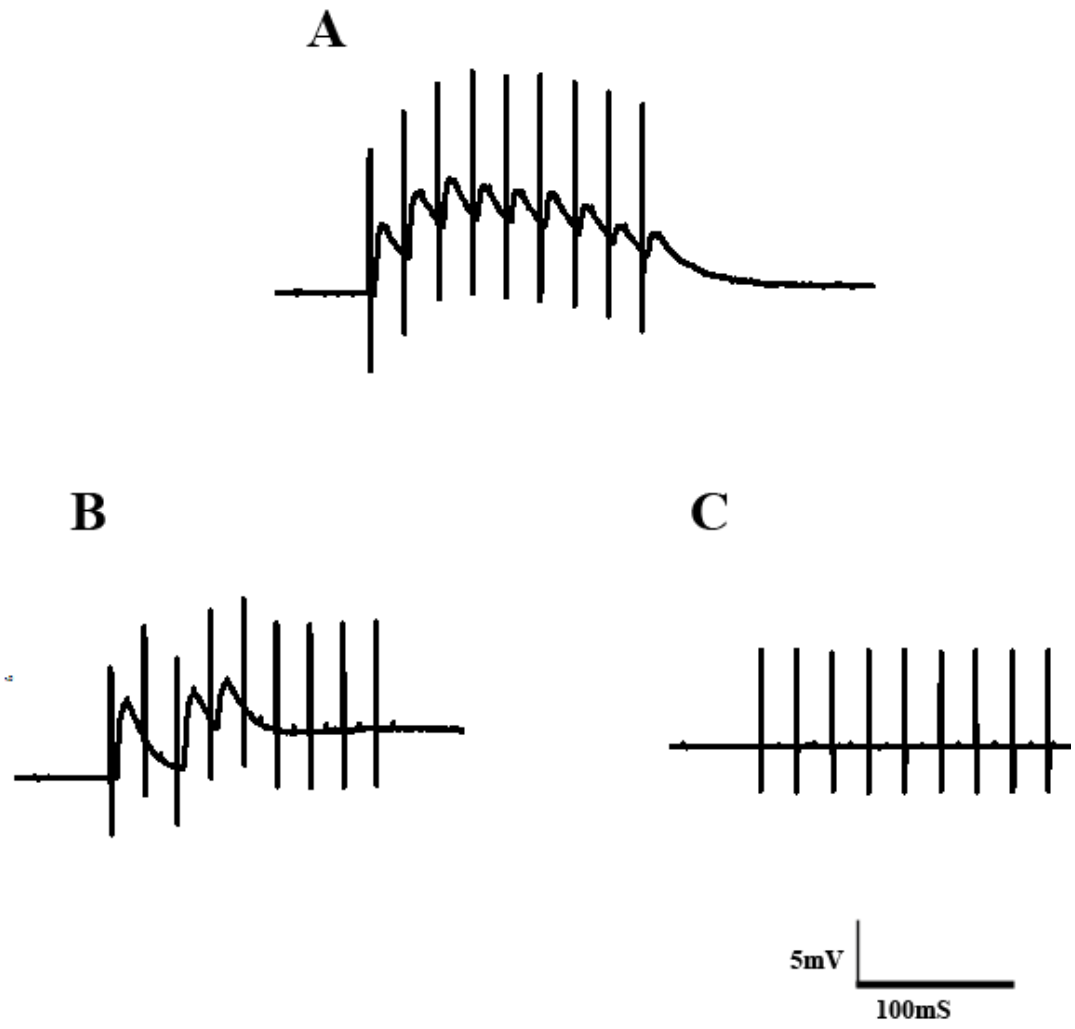
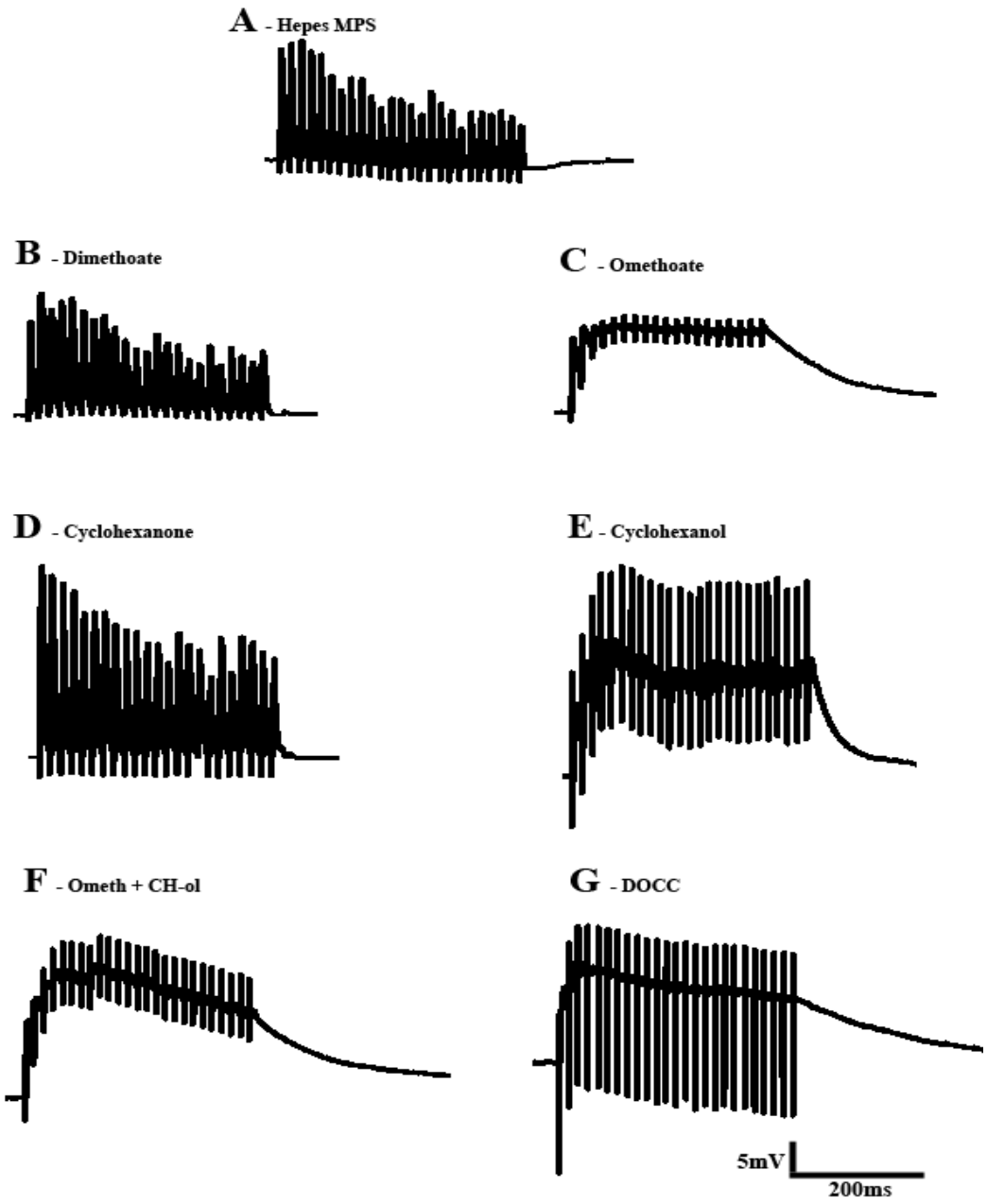

A
B
C
5mV
100mS

Fig 3.19: Traces from 50Hz trains (25 stimuli) and half decay time of last EPP response with different combination of pesticide components. A; Hepes MPS, B; dimethoate (1mM), C; omethoate (100µM), D; cyclohexanone (1mM), E; cyclohexanol (5mM), F; Omethoate and cyclohexanol, G; dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC), H; Half decay time (T50%) of the last EPP reponse in 50Hz trains of stimuli. Prolongation of last EPP decay was maximum at all four components together (trace G). Each bar represent mean ± SEM (n = 3 muscles muscles per each treatment group) ($p < 0.05$, **ANOVA, Bonferroni post test- treatements were compared with Hepes MPS**).


A - Hepes MPS
B - Dimethoate
C - Omethoate
D - Cyclohexanone
E - Cyclohexanol
F - Ometh + CH-ol
G - DOCC
5mV
200ms

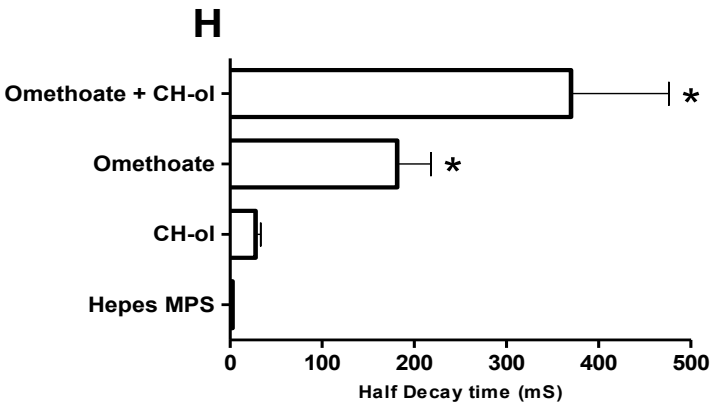
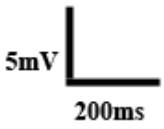

H
Omethoate + CH-ol
Omethoate
CH-ol
Hepes MPS
*
*
0
100
200
300
400
500
Half Decay time (mS)

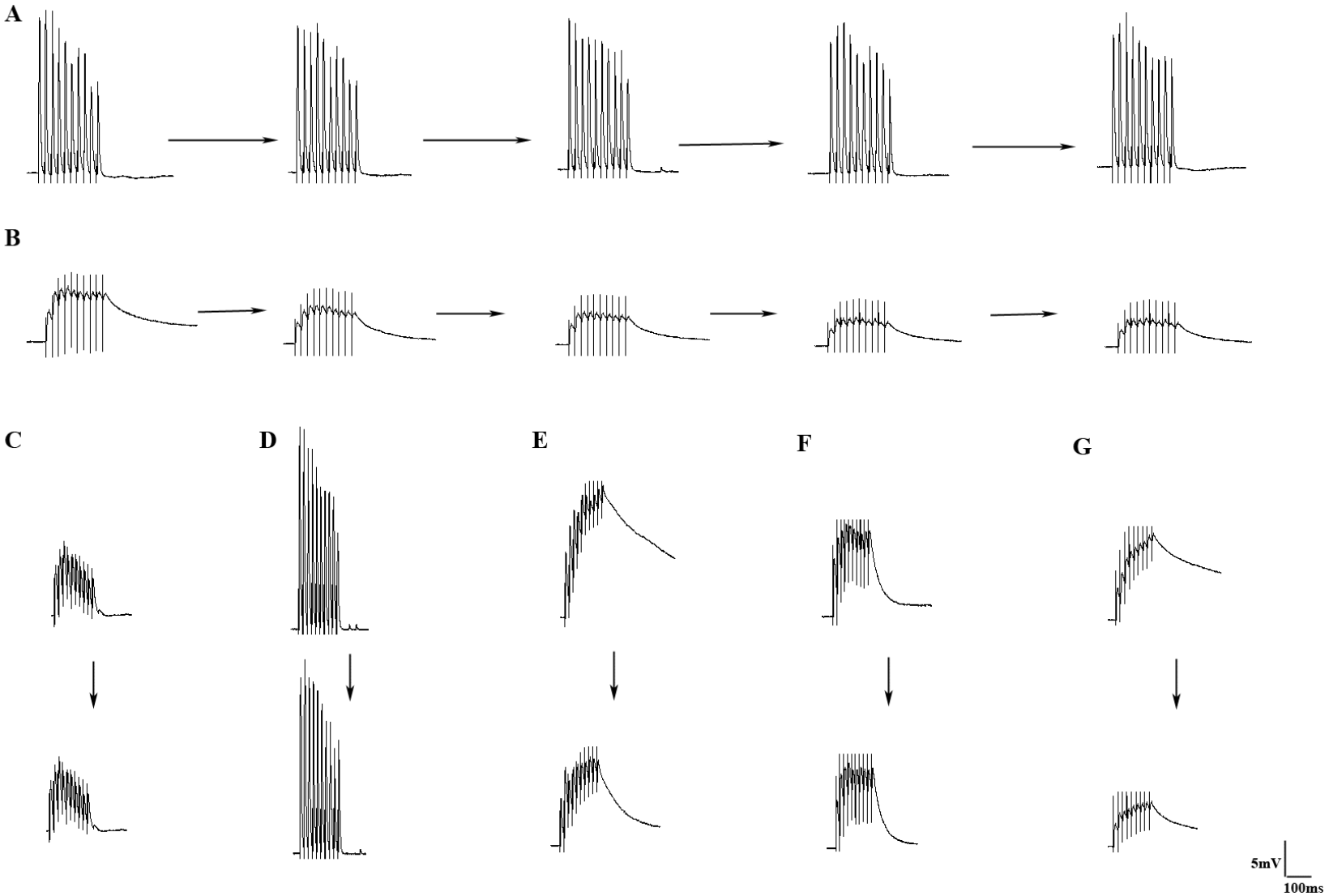
Fig 3.20: Traces from 50Hz trains (25 stimuli) demonstrating possible desensitisation of the AChRs. A: traces from a preparation treated with omethoate and cyclohexanol, B; traces from a preparation treated with dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC).

A - Ometh + CH-ol



B - DOCC

Fig 3.21: Multiple cycles of repetitive stimulation (50Hz) EPPs response trains (5s interval between each cycle) with different treatments. A: Hepes MPS, horizontal arrows indicate the subsequent cycles. There is no indication of fatigue or desensitization of EPP responses. B: DOCC, horizontal arrows indicate the subsequent cycles. There is dramatic fatigue along with desensitization with successive cycles. C – G: First and fifth responses of respective treatment. Vertical arrows indicate the response after 5 consecetive cycles C: Dimethoate, D: Cyclohexanone, E: Omethoate, F: Cyclohexanol, G: Omethoate and cyclohexanol. Dimethoate and cyclohexanone shows no fatigue or desensitization of EPP responses in subsequent repetitive stimulation while omethoate, and omethoate and cyclohexanol show clear fatigue or desensitization of EPP responses. Cyclohexanol alone also shows little fatigue or desensitization of EPP responses.


A
B
C
D
E
F
G
5mV
100ms

3.3.5. Metabolic breakdown products of dimethoate EC prolong muscle contractions

Finally, I asked what the functional effects of prolongation of the EPPs by individual components of pesticide might have on muscle contractions (twitches or tetani).

There was no evidence of selective loss of motor units with any of the treatments as delivery of graded stimulation before and after treatment produced no change in number of motor units per sample (Data not shown).

Analysis of the data revealed that dimethoate, cyclohexanone or cyclohexanol does not produce any modifications of twitch or tetanic tension compared to recordings in Hepes MPS (Fig 3.22, 3.23). There was slight twitch potentiation with omethoate and DOCC treated preparations (Fig 3.22, 3.23). In 2/3 experiments the twitch amplitude increased by more than 40% (with omethoate or cyclohexanol alone produced smaller increase: all three repeats, the maximal increase were about 10%). However, ANOVA indicated that the difference did not produce a statistical difference. The potential of type II statistical error in this experimemnts could perhaps be address in further experimets. However, since twitch amplitude was of less interest than the underlying synaptic physiology, no further twitch tension measurements were undertaken. Twitch amplitude percentages (twitch amplitude as a percentage of control twitch amplitude) with different treatments were observed at 0.1Hz frequency with reference to control MPS as follows: dimethoate 100.87±4.760, cyclohexanone: 91.48±1.40%; omethoate: 113.33±4.68%; cyclohexanol: 96.51±5.053%; omethoate and cyclohexanol: 148.21±34.16%; DOCC: 113.17±0.77% (mean ± SEM, $P > 0.05$, ANOVA, "$F = 2.05$") (These measurements presented as a percentages in order to standardize the data).

Summation of twitch responses was observed at frequencies as low as 1 – 2 Hz in preparations treated with combination of omethoate and cyclohexanol compared to other treatments (fig 3.24). The combination of omethoate and cyclohexanol also caused a smooth tetanic fusion at lower frequency (10Hz) than the individual components (Fig 3.25). However, incomplete tetanic frequency fusion was also evident with omethoate, cyclohexanol and DOCC preparations (fig 3.25).

Interestingly in preparations treated with cyclohexanol alone, 50Hz tetanic tension responses showed no increment in amplitude or the relaxation phase of the muscle contraction compared to that of Hepes MPS (Fig 3.26). However, omethoate-treated preparations produced a strong after contraction with slow relaxation after 50Hz stimulation (Fig 3.26).

Remarkably, but consistent with EPP data (in section 3.3.4) combination of omethoate and cyclohexanol produced an even greater prolongation of both after-contraction and the relaxation phase, lasting for several seconds (Fig 3.26).

Surprisingly, DOCC produced shorter and weaker after-contractions and a shorter relaxation phase compared to combination of omethoate and cyclohexanol together (Fig 3.26, 3.27). This was consistently observed with all three muscles tested with DOCC. Thus it appears that dimethoate and cyclohexanone partially counteract the response to omethoate and cyclohexanol. Besides the more rapid relaxation of the tetanic tension produced by of all four components together, these response amplitudes were also reduced compared to those in control MPS (fig 3.26). There are several possible explanations for this effect of DOCC, including desensitisation of the AChRs by accumulated ACh in the synaptic cleft due to anticholinesterase activity, thus curtailing the NMJ transmission. Alternatively depolarization block might have caused inactivation of the muscle voltage gated Na^+ channels leading prevention of AP propagation. Another possibility is the direct action of these components on the muscle fibre itself, hence minimising the ability to produce sustained prolonged contraction of the muscle. A third possibility is that dimethoate competes with omethoate reducing the inhibitory effects of the latter on AChE activity. However, this last explanation is not supported by the EPP data shown in Fig 3.12.

Direct stimulation of the muscle was used to test whether the effects of omethoate in prolonging muscle contraction were due to direct effects on muscle, rather than dependent on neuromuscular transmission. Direct stimulation bypasses the post synaptic ACh receptors and directly excites the muscle to produces an action potential and muscle contraction. However, direct stimulation also excites motor axons. Therefore to rule out a direct effect of omethoate on muscle contraction itself, I blocked AChR with d- tubocurarine. Direct stimulation of the muscle in the presence of 5µM d-tubocurarine (d-Tc) and omethoate (100µM) displayed no prolongation of the tetanic contraction compared to the effects of nerve stimulation at 50Hz stimuli (Fig 3.28). Thus, the prolonged tetanic contraction observed in the indirect stimulation is not consequence of the OP components affecting muscle contraction. The prolonged contraction observed in omethoate is therefore due to an effect on neuromuscular transmission.

In summary, the results in this Chapter suggest that omethoate and cyclohexanol, the metabolic breakdown products of dimethoate Ec, produce potent inhibition of AChE activity and additive effects on EPP decay. This leads to additive effects and profound disturbance in

muscle physiological function, including, prolonged after-contractions and slow relaxation in response to repetitive stimulation (table 3.3). However, I made a number of puzzling and confounding observations: for instance, high frequency repetitive stimulation with DOCC produced longest decay time in EPP responses; however the mechanical response to high frequency stimulation in DOCC is less affected than in a solution containing added omethoate and cyclohexanol alone.

Fig 3.22: Example traces comparing potentiation of twitch amplitude with different treatments compared to control MPS. Paired traces of tension represent control Hepes MPS trace (1st trace) and after 20 minutes incubation of respective treatment (2nd trace). Preparations were continuously stimulated at 0.1Hz stimulation frequency during 20 minutes incubation period. A: dimethoate (1mM), B; cyclohexanone (1mM), C; omethoate (100μM), D; cyclohexanol (5mM), E; Omethoate and cyclohexanol, F; dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC). Note that the twitch tension amplitude facilitation is relatively increased in combination of omethoate and cyclohexanol treated preparation, compared to other treatments.

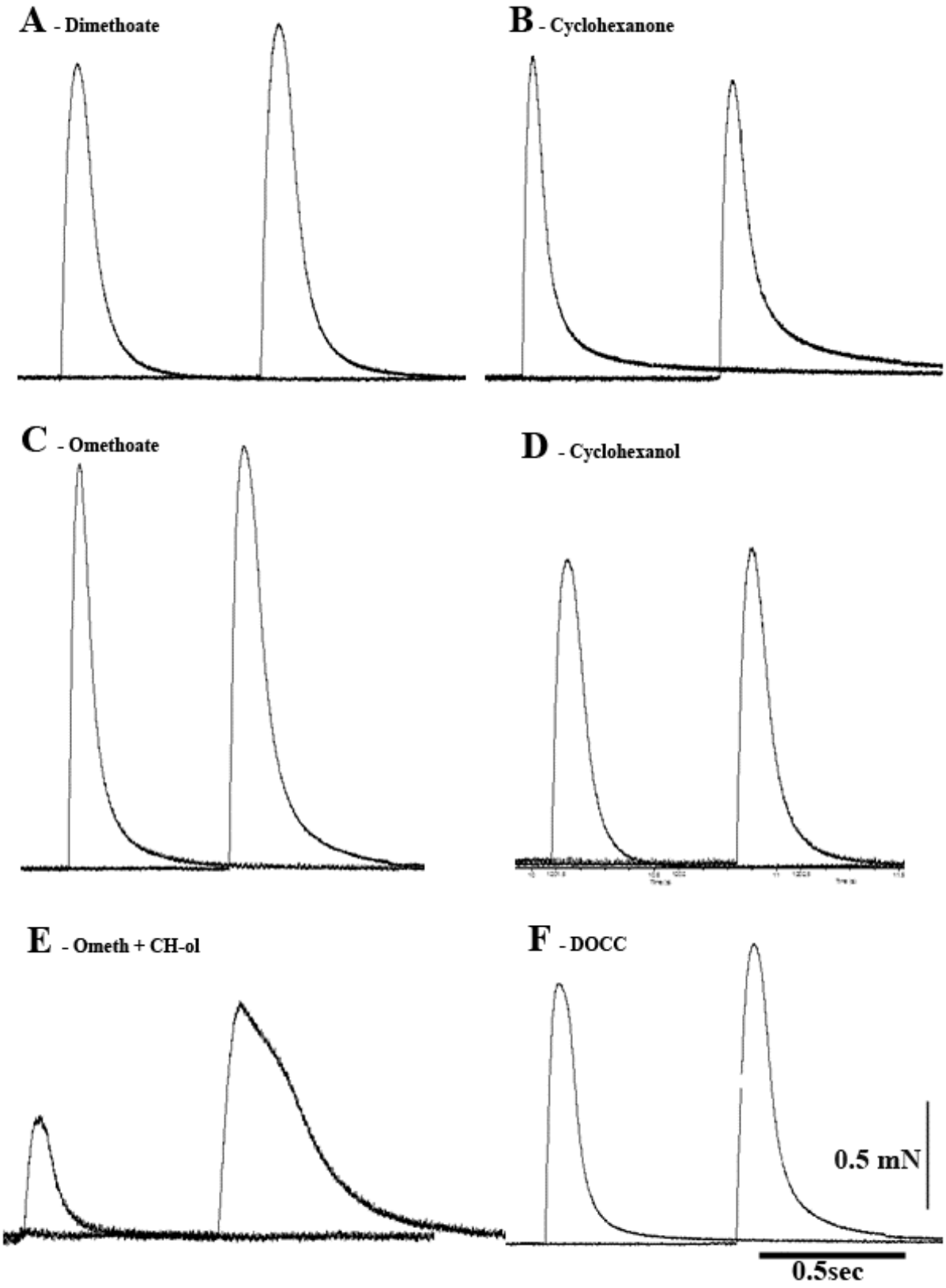

A - Dimethoate
B - Cyclohexanone
C - Omethoate
D - Cyclohexanol
E - Ometh + CH-ol
F - DOCC
0.5 mN
0.5sec

Fig 3.23: Comparison of twitch potentiation with different combinations of the pesticide components. A: Change in twitch amplitude over 20 minute (incubation period) after treatment at 0.1Hz stimulation frequency. Each point represent mean (n = 3 muscles in each treatment). B; Twitch amplitude % change after 20 minutes incubation with treatment. (Twitch amplitude% was calculated as a percentage of subsequent twitch amplitude from the first twitch amplitude (Control MPS)). Note that the combination of omethoate and cyclohexanol produces considerable increases in twitch amplitude compared to other treatments. Each bar represents mean ± SEM (n = 3 muscles in each treatment group) ($p > 0.05$, **ANOVA - treatements were compared with Hepes MPS).**

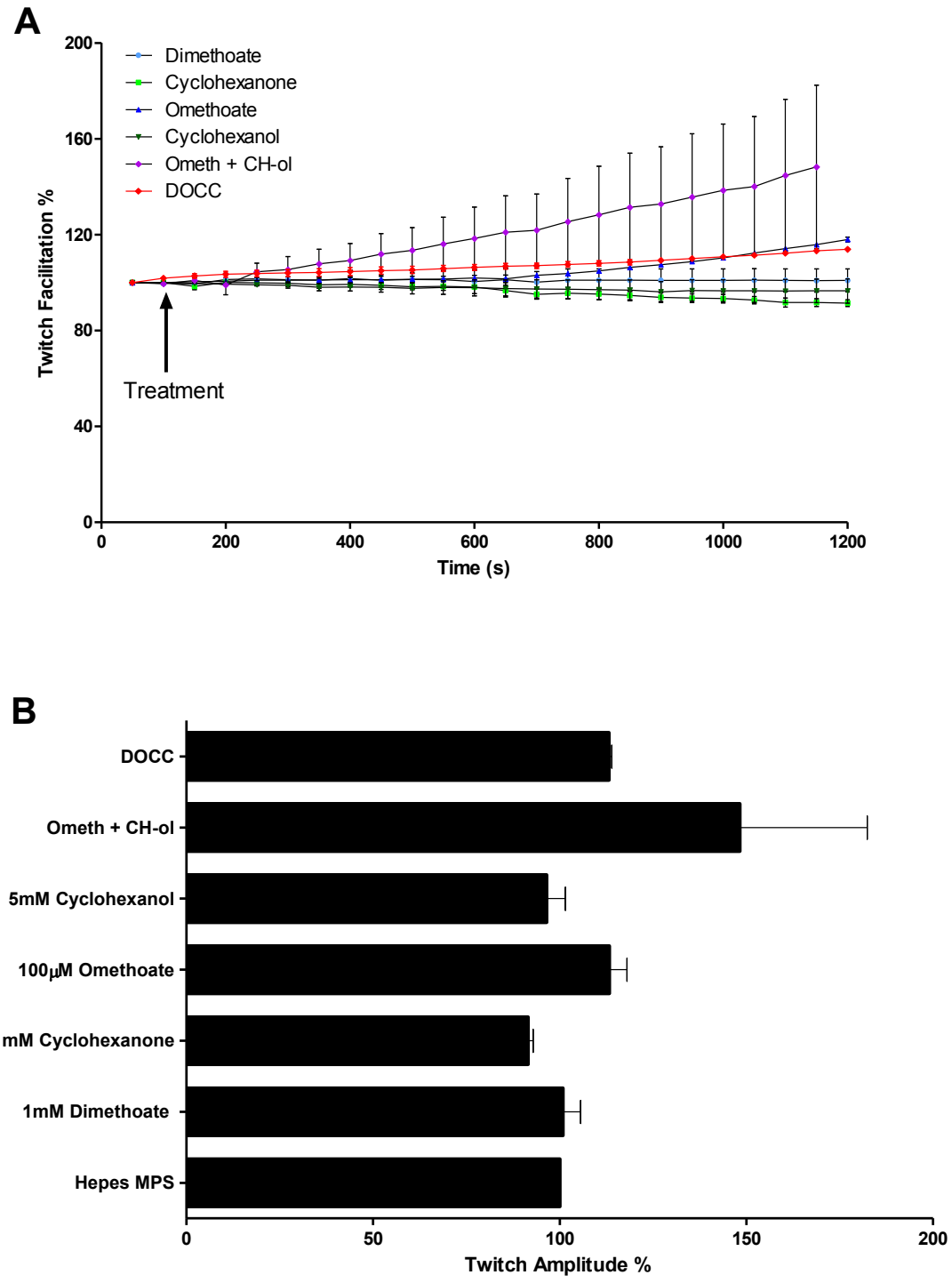

A
Dimethoate
Cyclohexanone
Omethoate
Cyclohexanol
Ometh + CH-ol
DOCC
Twitch Facilitation %
Treatment
Time (s)
B
DOCC
Ometh + CH-ol
5mM Cyclohexanol
100µM Omethoate
1mM Cyclohexanone
1mM Dimethoate
Hepes MPS
Twitch Amplitude %

Fig 3.24: Traces of twitch tension at low stimulation frequency (1Hz & 2Hz) with different combination of pesticide components. A; Hepes MPS, B; dimethoate (1mM), C; omethoate (100μM), D; cyclohexanone (1mM), E; cyclohexanol (5mM), F; omethoate and cyclohexanol, G; dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC). Note that the summation of repetitive stimulation response along with the base line shift is evident even at low frequencies (1Hz & 2Hz) with combination of omethoate and cyclohexanol treated-preparations.

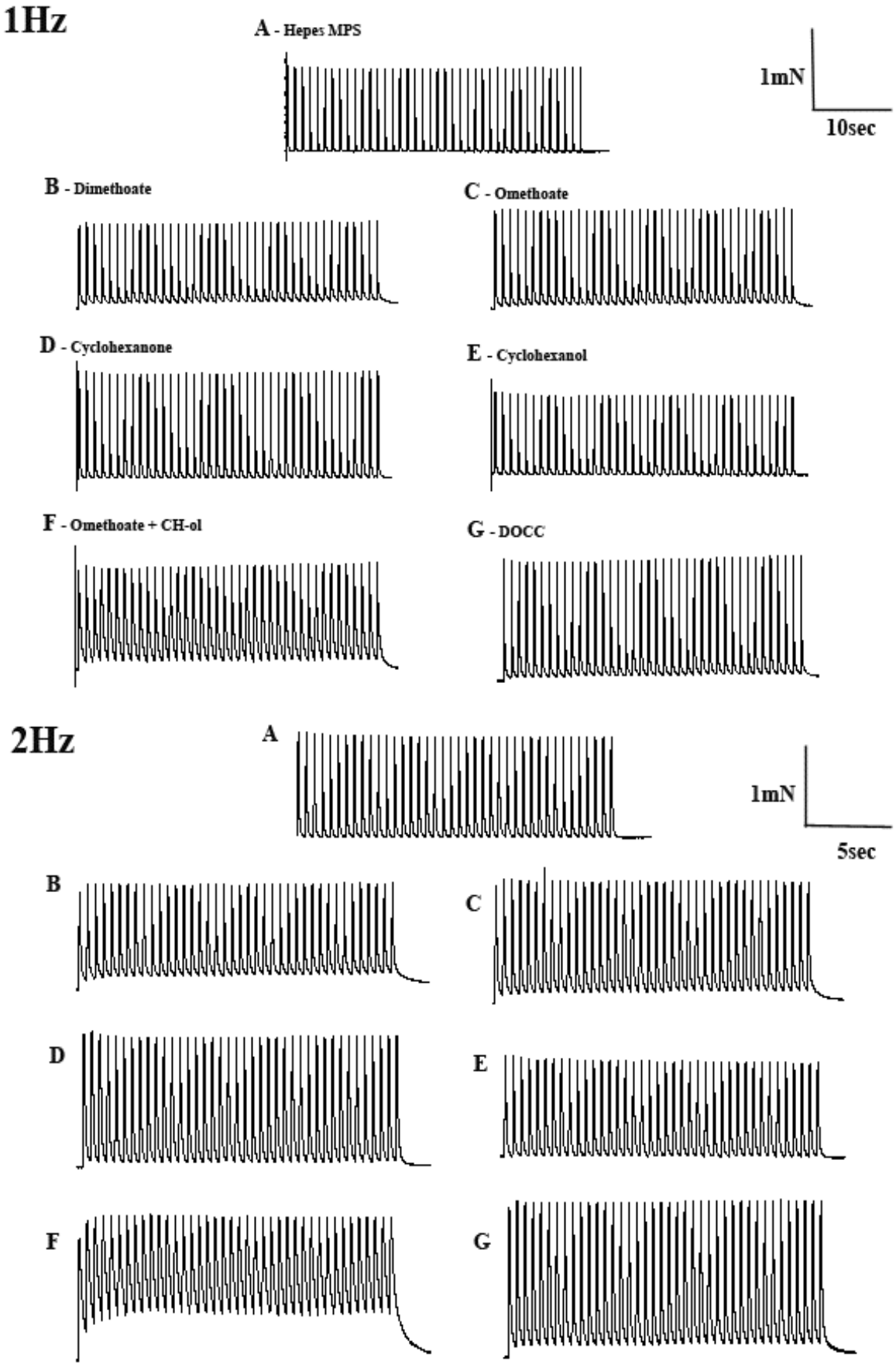

1Hz
A - Hepes MPS
1mN
10sec
B - Dimethoate
C - Omethoate
D - Cyclohexanone
E - Cyclohexanol
F - Omethoate + CH-ol
G - DOCC
2Hz
A
1mN
5sec
B
C
D
E
F
G

Fig 3.25: Temporal summation and tetani (10Hz) with different treatments. A; Hepes MPS, B; dimethoate (1mM), C; omethoate (100µM), D; cyclohexanone (1mM), E; cyclohexanol (5mM), F; omethoate and cyclohexanol, G; dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC). Note incomplete fusion at this frequency in all (C, E, F and G) but B and D compared to Hepes MPS (A). The most complete fusion at this frequency was observed with omethoate + cyclohexanol (trace F) and not DOCC (trace G), consistent with the data shown next in fig 3.26 & 3.27.

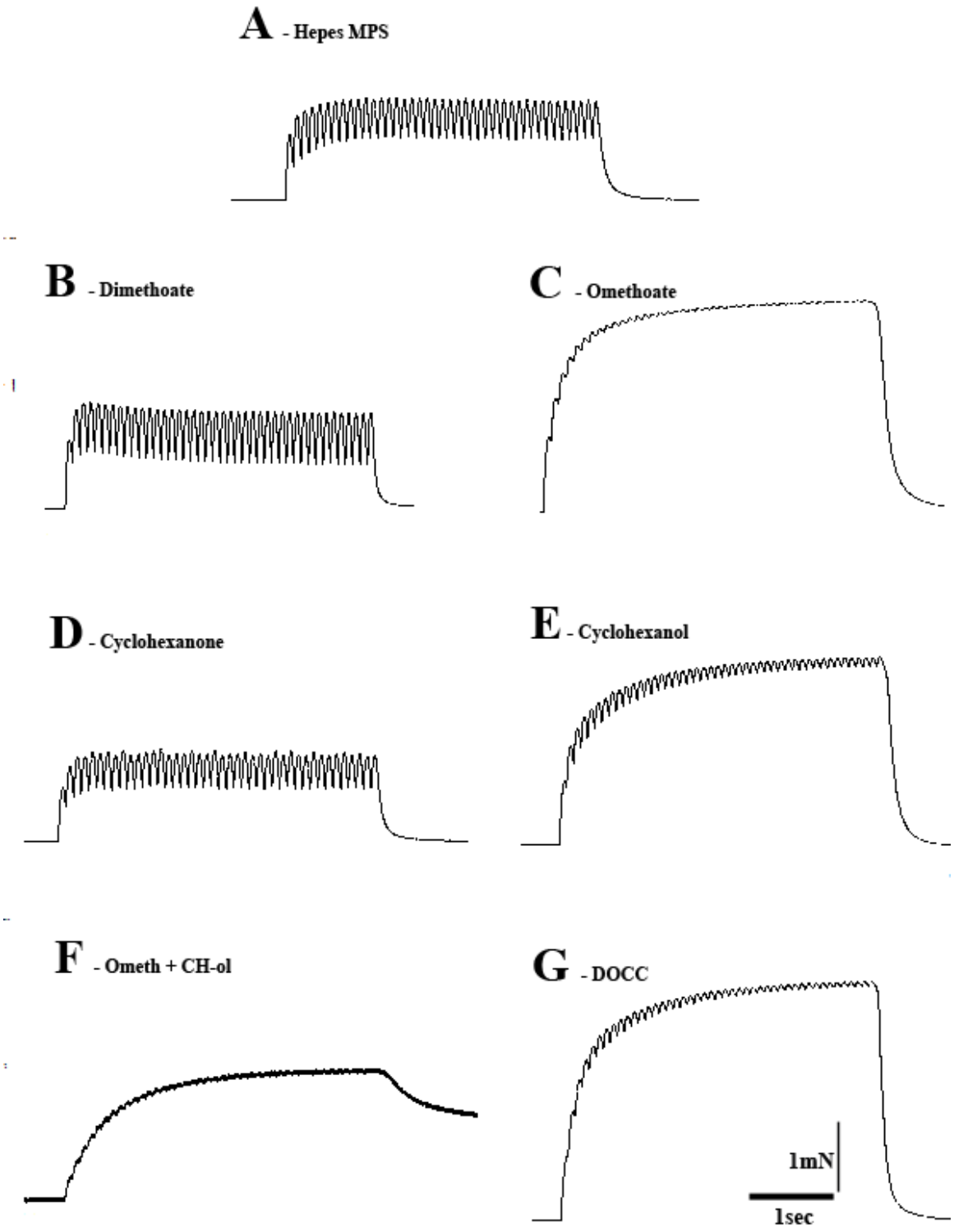

A - Hepes MPS
B - Dimethoate
C - Omethoate
D - Cyclohexanone
E - Cyclohexanol
F - Ometh + CH-ol
G - DOCC
1mN
1sec

Fig 3.26: Tetanic tension responses to repetitive stimulation 40 stimuli at 50Hz with different combinations of pesticide / metabolites components. The resonses to each treatment (red line) is shown for comparison with a control trace (Hepes MPS, 50Hz 40 stimuli - black line) obtained before adding the compound indicated. A: dimethoate (1mM), B; cyclohexanone (1mM), C; omethoate (100μM), D; cyclohexanol (5mM), E; omethoate and cyclohexanol, F; dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC). Note the prolonged after contraction that continued after a 50Hz stimuli train compared with control traces in panel C, E and F. Interestingly, the prolonged after contraction observed in the presence of omethoate and cyclohexanol (panel E) were reduced in duration when all four components were present (panel F).

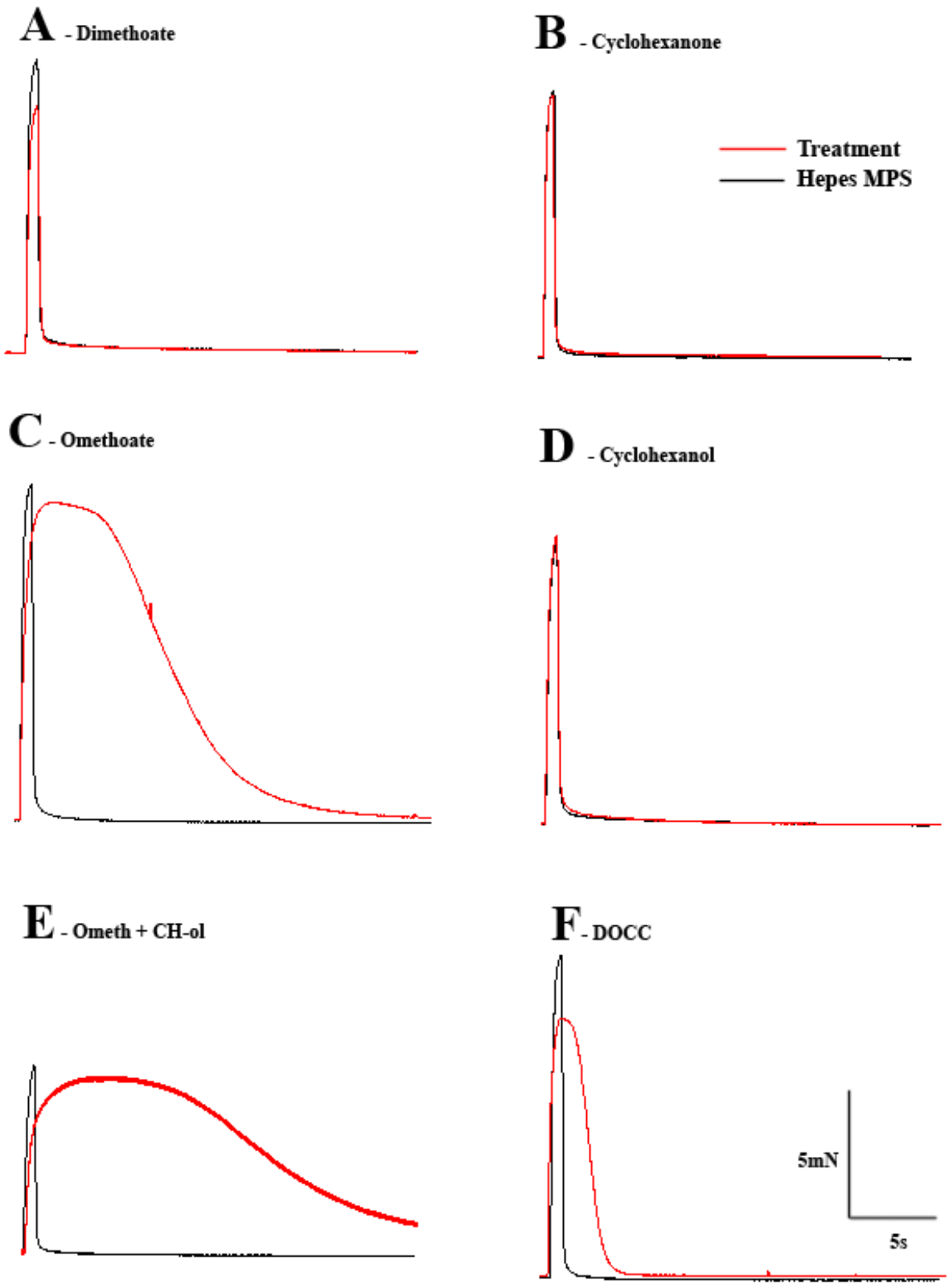
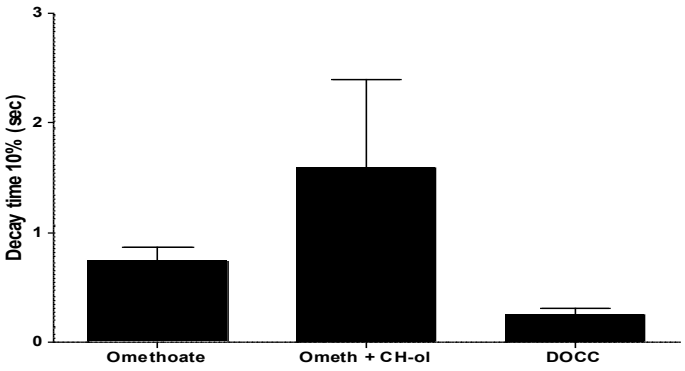
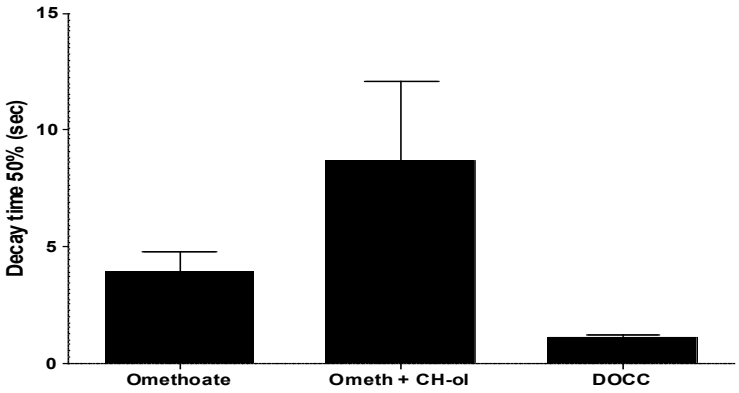

A - Dimethoate
B - Cyclohexanone
Treatment
Hepes MPS
C - Omethoate
D - Cyclohexanol
E - Ometh + CH-ol
F - DOCC
5mN
5s

Fig 3.27: comparison of 50Hz tetanic tension decay time (relaxation) with omethoate, Omethoate and cyclohexanol, and dimethoate, omethoate, cyclohexanone and cyclohexanol. A: 10% decay of the tetanic contraction, B; 50% decay of the tetanic contraction, C; 90% decay of the tetanic contraction. Note that the maximum decay of the tetanic contraction is produced by omethoate and cyclohexanol combination while DOCC produced the minimum. Each bar represents mean ± SEM (n = 3 muscles per each treatment group) ($p > 0.05$, **ANOVA- treatements were compared with Hepes MPS**).


Decay time 10% (sec)
3
2
1
0
Omethoate
Ometh + CH-ol
DOCC


Decay time 50% (sec)
15
10
5
0
Omethoate
Ometh + CH-ol
DOCC

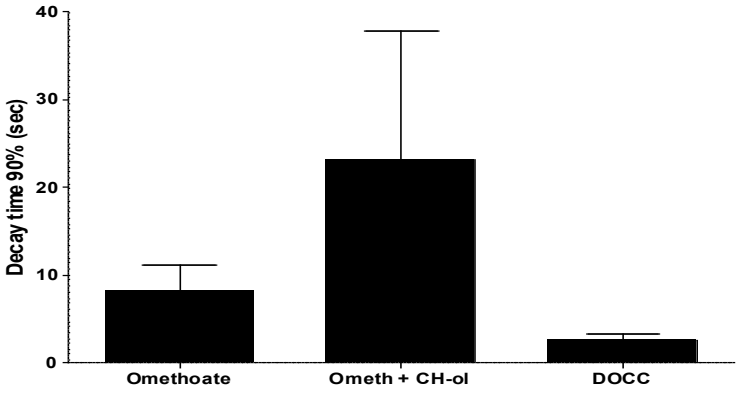

Decay time 90% (sec)
40
30
20
10
0
Omethoate
Ometh + CH-ol
DOCC

Fig 3.28: Comparison of direct and indirect tetanic tension (50Hz stimulation) in the presence of omethoate (100µM). Indirect stimulation produced a prolonged contraction with delay in relaxation, while direct stimulation (with d-tubocurarine) produced a rapid relaxation. This shows the involvement of ACh and post synaptic receptors in producing delay in relaxation of the muscle contraction with omethoate.

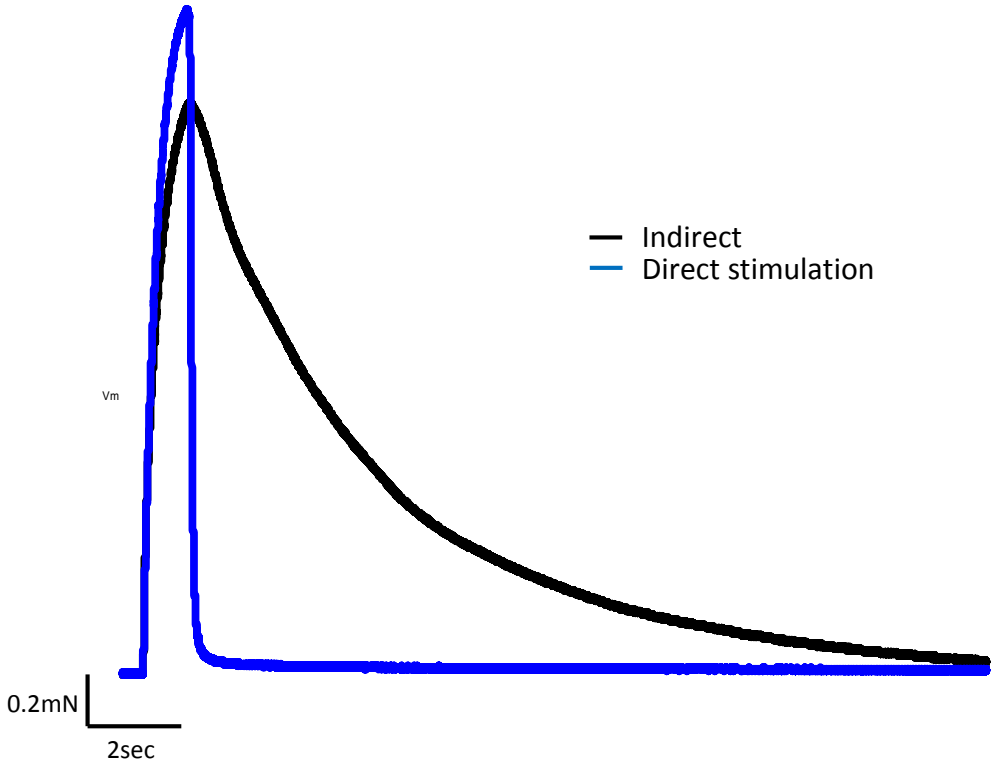

Indirect
Direct stimulation
Vm
0.2mN
2sec

Table 3.3: Summary of may findings in each experimental groups.

Experiment	Major Findings
EPP and MEPP recordings in samples treated with minipig plasma and neostigmine	Neostigmine treatment (positive control) significantly prolonged the decay time course of the EPPs and MEPPs ($p < 0.05$) Both pesticide plasma and dimethoate plasma treatments also significantly prolonged the decay time of EPPs ($p < 0.05$) consistent with AChE inhibition Neither Hepes MPS nor control plasma prolonged the decay time course of EPPs /MEPPs ($p > 0.05$) Pesticide plasma significantly increase spontaneous transmitter release (MEPP frequency) ($p < 0.05$) In addition pesticide plasma also produced reversible evoked transmission block (5/6 experiments)
EPP and MEPP recordings in samples treated with individual components of pesticide (dimethoate Ec) and their metabolites, either singly or in combination	Omethoate and cyclohexanol produced significant prolongation of EPP decay either singly or in combination ($p < 0.05$). However, combination of all four components and their metabolites (dimethoate, omethoate, cyclohexanone and cyclohexanol) yielded the longest EPP decay time Cumulative dose response curves indicated that low concentrations (μM) of omethoate have greater effects on EPP decay time, while dimethoate required higher concentrations (mM) for similar effects High frequency 50Hz stimulation produced tonic prolonged depolarization in omethoate, omethoate +cyclohexanol and DOCC treated preparations ($p < 0.05$)
AChE activity in muscle samples	100μM omethoate completely inhibited the AChE activity in muscle homogenates. Cyclohexanol (5mM) produced partial but significant inhibition of AChE ($p < 0.05$). Thus prolongation of EPP decay in omethoate + cyclohexanol cannot be explained by additive effects on AChE inhibition
Isometric contraction measurements in samples treated with individual components of the pesticide and their metabolites	Pesticide components and their metabolites either singly or in combination did not produce significant changes in twitch contraction amplitude ($p > 0.05$)

3.4 Discussion

Intermediate syndrome in OP pesticide poisoned human patients is a progressive muscle paralysis most notable in proximal muscles including respiratory and neck muscles. This muscle paralysis has a delayed onset compared to the "cholinergic crisis" observed soon after ingestion of OP pesticides. The mechanism underlying this respiratory muscle paralysis is hypothesised to be failure of synaptic transmission at the NMJ. Therefore the main objective of this chapter was to evaluate this hypothesis and to understand how pesticide components and their metabolites act on neuromuscular synaptic transmission, then to identify how these individual components either alone or in combination might contribute towards the failure of NMJ synaptic transmission. Hence, I hypothesised that neuromuscular transmission failure (i.e. respiratory paralysis) present in pesticide poisoned human patients is due to the combinatorial effects of both anticholinesterase and solvent. This was tested in this Chapter using different approaches including cellular responses (MEPP and EPP) and functional responses (twitch and tetanic muscle tension). I obtained substantial evidence to demonstrate that not only anticholinesterase compounds (dimethoate and its metabolite omethoate), but also the organic solvent (cyclohexanone and its metabolite cyclohexanol) are responsible for the observed alterations in NMJ synaptic transmission. I have also shown that compared to the parent components of dimethoate Ec (dimethoate and cyclohexanone), its metabolites (omethoate and cyclohexanol) produce effects at a substantially lower concentration, suggesting a more potent effects on NMJ synaptic transmission.

3.4.1 Pesticide plasma increases the time course of the synaptic transmission at the NMJ, and also produces reversible evoked transmission block

First, I tested my hypothesis using minipig plasma obtained from pigs treated with either dimethoate alone, commercial pesticide or saline. Use of minipig plasma instead of commercial pesticide was advantageous as plasma contained not only the parent compounds, but also its metabolites, at conceentrations that had been shown to cause NMJ failure (Eddleston et al., 2012).

My results with plasma treated nerve muscle samples show that control plasma has no effects on synaptic transmission. Neostigmine, as a positive control confirmed the classic anticholinesterase effects showing elongation of the decay phase of synaptic transmission of both MEPPs and EPPs (Blaber and Christ, 1967; Katz and Miledi, 1973; Kordas, 1977; Miledi et al., 1984). Both dimethoate plasma and pesticide plasma exhibited similar effects

to neostigmine in terms of increase decay time, suggesting the anticholinesterase effects. However the increment of decay time facilitation with treated plasma (dimethoate and pesticide) was not as great as with a low dose of neostigmine. This confounding effect could be due to; a) anticholinesterase activity is lower than expected in the sample, b) some of the molecules of anticholinesterases are bound to other molecules, or c) these samples (dimethoate and pesticide plasma) have a slow release mechanism of anticholinesterase molecules, i.e. OP; hence it is not available to act on the AChE enzyme (for example, some binding protein in plasma produce slow release) (For instance; lipid soluble OPs such as parathion tend to dissolved in body fat and release slowly to act upon acetylcholinesterase molecules). The possibility of a slow release mechanism is supported by the observation of the time taken to develop an increase in EPP decay time with different treatments. Neostigmine produced increase decay time within about 2 – 10 minutes of bath application, while dimethoate plasma and pesticide plasma took 30- 60 minutes and 2- 4 hrs respectively. However, even though pesticide plasma treated preparations had little effects on the time course of synaptic transmission, it eventually produced a complete evoked neuromuscular block.

Reduction in peak amplitude of the both MEPP and EPP was noted with neostigmine, while dimethoate plasma-treated preparations showed an increase in peak amplitude of both MEPPs and EPPs. Some reports showed increase in MEPPs amplitude of 1.5 to 2 fold with neostigmine (Boyd and Martin, 1956; Blaber and Christ, 1967), but this subsequently declines to control level, suggesting possibly desensitization or weak antagonism such as channel block of AChRs by the accumulated neurotransmitter present in the synaptic cleft (Eccles and Mac, 1949; Fatt, 1954). Furthermore there is also evidence of unchanged MEPP amplitude with AChE inhibition by paraoxon (Laskowski and Dettbarn, 1979). On the other hand, lower concentrations such as 10^{-9} - 10^{-7}M neostigmine, ambenonium and edrophonium increase the amplitude of EPPs by 0.5 - 2 fold (Blaber and Christ, 1967)while higher concentrations of neostigmine, physostigmine and edrophonium (>10μM) depress the increment of EPC amplitude (Albuquerque et al., 1988). I have used 10μM neostigmine in my experiments; therefore higher drug concentration might be a possible reason for observed amplitude reduction as in some of the previous reports (Albuquerque et al., 1988).

I also, observed significant depolarization of the resting membrane potential in neostigmine treated samples. This may also have contributed to the reduction in EPP amplitude, due to a reduction in driving force required produce additive synaptic depolarization. Some research has found that there is no significant change in resting membrane with anticholinesterase

treatment (Kuba et al., 1974; Tiedt et al., 1978; Bois et al., 1980; Tattersall, 1990), while some showed evidence of dose dependent depolarization by anticholinesterases (Blaber and Christ, 1967; Laskowski and Dettbarn, 1979) followed by spontaneous repolarization of the membrane potential (Meshul et al., 1985; Kawabuchi et al., 1991).

In addition, pesticide plasma also produced a significant increase in MEPP frequency suggesting increase in spontaneous release activity of neurotransmitter from presynaptic terminals. However, there were no discernible effects of any of the other treatments (neostigmine, control plasma and dimethoate plasma) suggesting this could be either due to a synergistic effect of anticholinesterase and solvent or solvent alone. It has previously been shown that frog muscle (Fatt and Katz, 1951) or rat diaphragm (Liley, 1956) treated with 1μM prostigmine showed no change in spontaneous transmitter release. However subsequent studies reports that micromolar concentrations of anticholinesterase stimulate spontaneous release (Blaber and Christ, 1967; Laskowski and Dettbarn, 1975; Bois et al., 1980)). It has also been suggested that MEPP frequency increases as a result of AChE inhibition and not through direct action of the drug on presynaptic terminal (such as action on potassium ion channels), as MEPP frequency increment was reversed using an AChE reactivator 2-PAM (Laskowski and Dettbarn, 1979). Hubbard et al (1968) showed that changing Ca^{2+} and Mg^{2+} concentration alters the MEPP frequency in mammalian preparation. However, if any compound acts directly on the mechanisms of spontaneous transmitter release at presynaptic terminal, it should not be affected by the altered Ca^{2+} and Mg^{2+} ion concentration of bathing medium. There is also evidence for inhibitory effects of AChE inhibitors on spontaneous transmitter release at frog NMJ (Duncan and Publicover, 1979). The authors showed that reduced Ca^{2+} entry into the presynaptic terminal causes a decrease in both spontaneous and evoked release. Even though, there is some evidence in the literature for increase MEPP frequency with anticholinesterase, I suggest that my results showing an increase MEPP frequency with pesticide plasma are more likely to be due to synergistic effects, of anticholinesterase and solvent or solvent alone, as dimethoate plasma has no effects on MEPP frequency.

More interestingly, my results suggested an evoked transmission block with pesticide plasma treated preparations after 2 – 4hrs post treatment, with failure to produce an EPP upon nerve stimulation (with intact MEPP). In previous reports it was noted a curare-like depression occurs with anticholinesterase-treated preparations (Eccles and Mac, 1949), and evidence for depolarizing effects with treatments were also documented (Nastuk and Alexander, 1954; Katz and Thesleff, 1957a; Blaber and Christ, 1967). There is also evidence for depolarizing

block effects of ambenonium and methoxyambenonium (anticholinesterases used to antagonized muscle relaxation) (Blaber 1960). In addition, irreversible neuromuscular block was observed with millimolar concentrations of paraoxon (Laskowski and Dettbarn, 1979). Surprisingly, however, when I treated FDB nerve-muscle preparations with a combination of dimethoate, omethoate, cyclohexanone and cyclohexanol at similar concentrations to known present in minipig plasma, I did not see any evoked transmission block. Since control plasma did not produce an evoked transmission block, I would suggest that pesticide plasma contains components other than dimethoate, omethoate, cyclohexanone and cyclohexanol that might have contributed to the observed transmission obstruction.

In pesticide poisoned patients, progressive respiratory failure, which has later onset (24 – 72 hrs post poisoning) and lasts up to several weeks, is the primary feature of the intermediate syndrome. However, reported transmission block with AChE inhibition, does not provide sufficient direct evidence for progressive neuromuscular transmission failure as a cause of muscle weakness. Moreover, available evidence on irreversible NMJ transmission failure caused by paraoxon (active form of parathion anticholinesterase) occurs at higher concentrations of this compound (millimolar). However, in human pesticide poisoned patients with respiratory paralysis, the plasma concentration of OP pesticide (active component) is around micromolar (5 - 100μM) (Eddleston et al., 2005; Eddleston et al., 2008; Eddleston et al., 2012). Pesticide absorption from the gut, metabolism in the liver and diffusion in-to the neuromuscular junction might have major contributions to this latter onset and progressive respiratory paralysis in humans. Furthermore, excretion of the pesticide via urine might also influence the final plasma concentration. Moreover, pesticide contains not only anticholinesterase, but other components such as solvent and surfactant, the synergistic action of these compounds and their metabolites might influence the final outcome of respiratory paralysis in human patients. Finally, hyperactivity of respiratory muscles during cholinergic syndrome due to hypoxia might also have acted as a user dependent block on development of respiratory paralysis.

Although I did not observe a neuromuscular transmission block with combination of dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC) in-vitro, reversible evoked transmission block was noted with the pesticide plasma experiments. This is most likely to be due to synergistic action of anticholinesterases, solvent and other components present in the minipig plasma. This synergistic action is also supported by the evidence shown in the minipig model of OP toxicity, as dimethoate alone did not produce any failure in mechanomyography, while commercial pesticide-treated pigs showed clear evidence for

transmission block (failure in mechanomyography, 12 hrs post poisoning) (Eddleston et al., 2012). This study also showed that there is no neuromuscular transmission block observed with minipigs treated with solvent alone, suggesting this transmission failure is more likely to be due to synergistic action of anticholinesterase and solvent.

3.4.2 Metabolic breakdown products of dimethoate Ec is more potent than their parent constituents

Next I investigated how the individual components of pesticide alone or in combination acted on the synaptic transmission at the NMJ.

The increased half decay time of EPPs evident at 1Hz with both dimethoate and omethoate is quite consistent with their known anticholinesterase activity. However, the observed increment in half decay time of EPPs of cyclohexanol treated preparations is most likely to be a result of complex mechanism other than inhibition of cholinesterase activity. The rationale behind this speculation comes from the additive effects on half decay time with combination of omethoate and cyclohexanol. The AChE activity in muscle homogenates treated with either omethoate or cyclohexanol has shown that 100μM omethoate produced 99% inactivation of AChE (Fig. 3.13). Although cyclohexanol (5mM) alone produced 25 – 30% inactivation of AChE enzyme, if cyclohexanol was to increase half decay time through anticholinesterase activity, I would not have observed any additive effects when combined both together, because by 100μM omethoate would inhibited almost all the AChE enzymes in the sample. Therefore this suggests a more complex mechanism of action by cyclohexanol on NMJ. For instance, the effects could be similar to those reported for ethanol on mouse NMJ, which enhances synaptic transmission at the NMJ via its action on nicotinic ACh receptors. Ethanol increases the open channel time of the receptors. In addition ethanol potentiates neurotransmitter release, mediated by inhibition of the delayed rectifier potassium currents at the presynaptic terminal.

The observed reduction in peak amplitude with dimethoate (1mM) and omethoate (100μM) tallies with the previous literature as it has shown that higher concentrations of neostigmine, physostigmine and edrophonium (>10μM) depress the increment of EPC amplitude (Albuquerque et al., 1988). Inhibition of AChE results in accumulation of ACh molecules in the synaptic cleft, thereby triggering activation of the AChRs repeatedly. This will lead to reduction in the probability of post-synaptic membrane mediator (ACh) sensitivity, i.e. desensitization will occur, thereby reducing the EPPs amplitude. Receptor desensitization

with acetylcholinesterase inhibition (Katz and Miledi, 1977; Wray, 1981; Akaike et al., 1984; Shaw et al., 1985; Sherby et al., 1985) including evidence of NMJ transmission failure via desensitization of the post synaptic nicotinic receptors is well documented (Katz and Thesleff, 1957a; Thesleff, 1959; Katz and Miledi, 1973; Akasu and Karczmar, 1980; Giniatullin et al., 1997; Giniatullin and Magazanik, 1998). More surprisingly, cyclohexanol treated samples also showed a significant reduction in peak amplitude. Taken together, with the increased excitable threshold to produce an EPP from cyclohexanol treated preparations, I suggest that the EPP amplitude reduction may be due to a presynaptic mechanism of action, in addition to its post synaptic effects (such as desensitization of ACh receptors). Presynaptic effects could include, for example, effects on the nerve terminal currents (Ca^{2+} or K^{+}), reducing exocytosis. Possible post synaptic involvement of cyclohexanol include modulation of passive membrane properties of muscle fibres such as changes in input resistance, action potential threshold etc.

My cumulative dose response curves show that metabolites of both dimethoate and cyclohexanone have more potent effects on NMJ synaptic transmission, confirming previous observations of omethoate and cyclohexanol effects on NMJ synaptic transmission.

In the reaction step of phosphorylation and inactivation of the AChE molecule, the X moiety is displaced from the phosphorus atom (OP compound) by a serine hydroxyl group (Fig 1.6). According to one view the kinetics of this reaction is determined by the X moiety (leaving group) (Sidell and Borak 1992). Both dimethoate and omethoate have the same X subgroup (thio-N-methylacetamide group) (Fig 1.7), therefore both of them should show same efficacy in inactivating the enzyme. However, phosphorothioate compounds (e.g. dimethoate) require bio-activation to their phosphate analogue (i.e.omethoate) in order to produce more efficient inactivation of the AChE enzyme. Therefore omethoate produces more potent effects at lower concentration on NMJ transmission compared to dimethoate. (See Lucier & Menzer, 1970)

On the other hand, the mechanism of increase potency of cyclohexanol compared to cyclohexanone on synaptic transmission at the NMJ is yet to be investigated, as there is no clear evidence in the literature.

3.4.3 Omethoate and cyclohexanol produce synergistic effects on neuromuscular transmission

Next I turned to investigating how activity might modulate the synaptic transmission in the presence of pesticide components.

High frequency repetitive stimulation responses showed possible evidence on short term synaptic transmission depression (fig 3.21) and desensitization (fig 3.20) with DOCC treated preparations. It had also shown that the combination of omethoate and cyclohexanol produced more pronounced effects than these components alone. As I have mentioned earlier, muscle paralysis is more prominent in respiratory muscles of pesticide poisoned patients indicating possible influence of use dependent transmission block at NMJ in the presence of pesticide components. Therefore, results from high frequency repetitive stimulation could enable us in the future, not only to understand which components of pesticide are responsible of failure of synaptic transmission, but also constitute a simple *in-vitro*, assay which could be used to test treatment of synaptic transmission failure at NMJ following OP pesticide poisoning in humans.

Finally I have tested the hypothesis that the combination of omethoate and cyclohexanol produce synergistic effects on using a more functional aspect of NMJ synaptic transmission, namely nerve-evoked muscle contraction.

Evidence of twitch amplitude observed with anticholinesterases (dimethoate, and omethoate and its combinations) treated preparations confirms the results from previous studies (Barnes and Duff, 1953) (see page 125 and fig 3.22 & 3.23). However, there are some discrepancies over tetanic tension responses with the treatments. Previous reports suggest that low doses of OP compounds produce depressed tetani at different frequencies depending on the muscle fibre types. Brimblecombe (1970b, a) showed that low doses of sarin applied to slow twitch muscle (soleus) caused depression of tetani at 60 Hz frequency, while fast twitch muscle (flexor hallucic longus / FHL) sustained the tetani until 150 Hz frequency. However higher doses of sarin produced depression of tetani at all frequencies (both FHL and soleus), while tetanic response waned rapidly to zero at higher frequencies. Yet, our findings did not show a depressed tetani even at higher frequencies. For this we may need to take into account the fact that FDB muscle may be a mixed muscle containing both fast and slow twitch muscle fibres which are also short in length. Nevertheless, I observed prolonged sustained contractions in omethoate (either alone or in combination) treated preparations. It is also evident that very little activity of AChE is needed to show a sustained response to tetanus

(Barnes and Duff, 1953). Therefore it might be possible that the amount of AChE inhibition, together with the muscle fibre type, has a role to play in the discrepancies of tetanic responses I observed with my preparations. Consistent with the EPP data, the most significant alteration of muscle contraction was observed with the combination of omethoate and cyclohexanol. Surprisingly, DOCC produced less pronounced effects in muscle contraction compared to a combination of omethoate and cyclohexanol.

Even though there are previous reports about the effect of anticholinesterase activity on NMJ synaptic transmission and muscle contraction, there is less information on the effects of organic solvents. It has been shown that reduced twitch and tetanic tension occurs at fast and slow muscles treated with ethanol in *in-vitro* preparations (Taylor et al., 1992; Pagala et al., 1995). Similarly, high concentrations of ethanol depress NMJ contraction, while depression effects were inhibited faster when prostigmine administrated in to the sample (Etessami, 1972). It was shown that reduced NMJ contraction is via decreases intracellular calcium-ion transients (Cofan et al., 2000). Furthermore, dimethyl sulfoxide (DMSO) also reduces the muscle contraction (both twitch and tetanus) in dose dependent manner (Wali and Hayter, 1988; Reid and Moody, 1994; Velasco et al., 2003). However, there is no evidence on reduced muscle function with cyclohexanone or cyclohexanol in experiments. Therefore it is essential to further examine the mechanism of action of both pre synaptic and post synaptic alterations produced by pesticide solvent either alone or in combination with anticholinesterases.

Thus, I set to investigate further the synaptic mechanism using these pesticide components in my next chapter.

Chapter 4: Further analysis of mechanisms of action of Dimethoate EC pesticide and its metabolites on neuromuscular transmission

4.1 Introduction

After initial characterization of the EPP properties, from the early 1950's investigators began to explore the underlying current behaviour in neuromuscular preparations in response to anticholinesterases. Voltage clamp recordings of endplate currents (EPC's) in the presence of an anticholinesterase (eserine) were first described by the Takeuchi and Takeuchi (1959), who demonstrated a prolongation of both EPC rise time and half decay time. Kordas et al (1972; Kordas et al., 1975) showed that at higher concentrations of the anticholinesterases (eserine and prostigmine) EPC has complex decay kinetics since they could no longer can fitted by a single exponential function, compared to untreated preparations. Later, it was also shown that irreversible anticholinesterases such as OP compounds, also showed double-exponential decay of EPC: as well as significant prolongation of decay (Pascuzzo et al., 1984). Interestingly, Aguayo and Albuquerque (1986) also demonstrated a double exponential decay function when preparation treated with 1-phenyl-4-piperidino-cyclohexanol. This raises the intriguing question whether cyclohexanol itself, the solvent metabolite of Dimethoate Ec, might also have complex effects on the decay time of EPCs.

Initial evidence of effects on passive membrane properties with anticholinesterase (non OP) was first reported by Blaber (1972), who described no discernible effects on input resistance or equilibrium potential in edrophonium-treated preparations. However, later reports on the effects of the OP compound ecothiopate showed reduction in the input resistance of muscle fibres (Kelly and Ferry, 1994).

The basic physiological mechanism of neurotransmitter release (that is, the quantal nature of neuromuscular transmission) was first established by Katz and his colleagues in the 1950s' are who made the first intracellular microelectrode recordings of MEPPs and EPPs (Fatt and Katz, 1951; Del Castillo and Katz, 1954, 1956). Since then, effects of anticholinesterase (OP and non OP compounds) treatment on both spontaneous and evoked transmitter release from presynaptic terminals of neuromuscular junction have been described (Straughan, 1960; Laskowski and Dettbarn, 1975). However, the non-linearity of EPP responses (McLachlan and Martin, 1981), argues for use of EPC to estimate quantal contents because EPCs are evoked at a constant membrane potential, thus the driving force is constant and the magnitude of the EPCs is directly proportional to the quantal content.

In the previous Chapter, I evaluated the change in EPP and MEPP characteristics with pesticide components and their metabolites, either singly or in combination, and their consequences for twitch and tetanic contractions of muscle. I observed that the metabolites

omethoate and cyclohexanol produced greater effects at a substancially lower concentration at both cellular and functional levels compared with their respective parent compounds dimethoate and cyclohexanone. In addition to the anticholinesterase effects I observed with omethoate, I detected significant effects with cyclohexanol including prolongation of EPP decay, changes in the excitability of nerve, and synergistic effects in combination with omethoate on both EPPs and muscle contractions. In order to understand better the mechanisms underlying these combined effects of omethoate and cyclohexanol, I turned my attention to the effects of these compounds on the underlying endplate currents.

First, however I asked whether these compounds have any effects on passive membrane properties of the muscle fibres. Changes in excitability (action potential threshold and current required to produce an action potential) of the muscle fibre along with the passive membrane properties (input resistance and capacitance) would give rise to a plausible explanation for some of the observed EPP and tension record characteristics described in the previous chapter. Secondly, I asked whether EPC in the presence of omethoate or cyclohexanol (either singly or in combination) have similar physiological characteristics to those I observed for EPPs. Thirdly, I sought to measure neurotransmitter release (quantal content) from presynaptic terminals in omethoate and cyclohexanol-treated preparations. For this I used both direct and variance methods of quantal analysis (See General Introduction). Lastly, I have also attempted to explain the unexpected effects of cyclohexanol, including possible effects on presynaptic currents and ACh transport into synaptic vesicles.

4.2 Methods

The following methods are specific to this chapter. For general methods and chemicals, refer to Chapter 2.

Base line recordings for all the experiments were carried out in Hepes MPS at room temperature unless otherwise stated on specific occasions. The majority of my experiments were carried out using intercostal nerve-trangularis sterni (TS) muscle preparations, however in some occations tibial nerve, FDB muscle preparations were also used (quantal analysis using the Variance method).

4.2.1 Input resistance measurements

Microelectrodes (Harvard apparatus / glass capillary tube, GC150F-15, 1.5 O.D. * 0.86 I.D.) for both intracellular recording electrodes and current passing electrodes were pulled using a

Flaming / Brown micropipette puller. Recording electrodes were back filled with 4 – 5M potassium acetate solution and had microelectrode tip resistance of 30 – 50 MΩ. Current passing electrodes were back filled with 3M KCl solution and had microelectrode tip resistance of 5 – 10 MΩ. Single muscle fibres were identified with the use of upright microscope (Axioskop 2, 40X-W/0.75 numerical aperture water-immersion objective; Zeiss, Oberkochen, Germany). Voltage and current microelectrodes were mounted (Sutter instruments MP285 electric micromanipulators) and tips were separated 50 – 100 μM apart on impalement of muscle fibres at the endplate. Voltage recording were obtained using a HS2A X 0.1H head stage connected to Axoclamp 2B amplifier while step currents were delivered to a HS2A X 1H head stage connected to the same amplifier. Signals were further amplified using Neurolog NL106 AC/DC amplifiers, low pass filtered at 3KHz (NL92) and then passed through a Humbug filter to eliminate 50 Hz main interference. Then they were digitized (20KHz) in to a personal computer (DELL., USA) via Digidata 1322A (Axon instrument, USA) interface. The cells were depolarized or hyperpolarized to a -70mV by passing constant current to standardize the measurement. In some instances, they were hyperpolarized to -90mV where none of the fast, voltage-sensitive Na channels in the muscle membrane are inactivated and the rate of rise of the action potential is maximal (Grampp et al., 1972; Harris and Marshall, 1973). A series of 100ms square wave current pulses were passed in 10nA steps to the muscle fibre via the current electrode and corresponding voltage displacement was recorded using Clampex software (pClamp 9 software; Axon instrument, USA). The input resistance was calculated as the slope of the current-voltage (*I-V*) plot using the Clampfit program (pClamp9 software, Axon Instruments), in the region where the response were linear. Recordings with evidence of activation or inactivation of outward and inward rectifying potassium channels at large depolarization or hyperpolarization were excluded.

4.2.1.1 Technical challenges with input resistance measurements

Activation and inhibition of inwardly rectifying potassium channels influenced responses to step current injection, resulting in a non-linear relationship between current and voltage (Fig 4.1 A,B). Another notable complication was a ragged response to depolarising current injection, perhaps partly due to asynchronous activation of delayed rectifying potassium currents. This was usually as a result of poor current-passing electrode characteristics. This also became problematic when I measure the action potential threshold (fig 4.1 C). This problem was overcome by using current passing electrodes that were less than 10MΩ resistance.

4.2.1.2 Sampling protocol for Input resistance measurements and time constant

Base line recordings for input resistance measurements were made from 10 fibres (trangularis sterni muscle preparations) followed by adding respective drugs in to the bath to achieve the targeted drug concentration. Samples were incubated for 20 minutes to achieve equilibrium followed by further measurements of input resistance in 15 fibres, to evaluate the effects of treatment.

4.2.2 Action potential threshold and amount of current required to produce an action potential

Action potential threshold was defined as the voltage at the (V_t) inflexion/saddle point of the rising phase of the endplate potential - action potential complex, at the time T_1 when the rate of the rise increased significantly to produce an overshoot of the action potential. The threshold current required to trigger an action potential in response to step currents, was recorded. (see inset fig 4.6A).

4.2.3 EPC recording (Indirect stimulation) using two electrode voltage clamp (TEVC)

Intercostal nerve, trangularis sterni muscle preparations were pre-treated with μ-CTX GIIIB to block muscle action potentials. Nerve – muscle preparations were then mounted in a Sylgard lined Petri dish / recording chamber which contained Hepes MPS at room temperature. Thin-walled glass microelectrodes (Harvard apparatus / glass capillary tube, GC150TF-10, 1.5mm O.D. X 1.17mm I.D.) for intracellular recordings were pulled using P87 Flaming / Brown micropipette puller and back filled with either 4 - 5M potassium acetate (KAc) (voltage electrode; tip resistance 5 - 15ΩM) or 3M potassium chloride (KCl) (current electrode; tip resistance 1 - 5ΩM). Low impedance recording and current electrodes were crucial in achieving adequate voltage clamp as this helped to reduce noise and improved the voltage clamp frequency response. The intercostal nerve was positioned in suction electrode and connected PG 4000A digital stimulator (Cygnus technology, Inc., USA) for the simulation, which was driven by PClamp software via digidata 1322A interface. Single muscle fibres were impaled with both voltage and current electrodes at either side of an endplate with the separation of 50 – 100 μM. Accurate impalement was assisted by observing the tip through an upright microscope (Axioskop 2, 40X-W/0.75 numerical aperture water-immersion objective; Zeiss, Oberkochen, Germany). Voltage recording were obtained using HS-2A X0.1H head stage connected to Axoclamp 2B

amplifier while feedback currents were applied using a HS-2A X1H head stage connected to an Axoclamp 2B amplifier. A holding potential of -70mV was applied and maintained in voltage clamp mode. The gain of the voltage clamp amplifier was adjusted at every recording in an effort to achieve faithful clamp with a target of <10% escape from the unclamped voltage response, while preventing signal oscillation. The level of the MPS solution was also reduced to minimize the electrode – bath capacitive coupling, along with the shielding of the water-immersion optical lens above the meniscus with aluminium foil. Recordings were carried out at the room temperature (20 – 25 ºC) and bath temperature was monitored using digital thermostat. Recorded signals were also amplified using Neurolog NL106 AC/DC amplifiers, low pass filtered at 3 KHz (NL92) and then passed through a Humbug to eliminate 50 Hz interference. Then they were digitized in to a personal computer via Digidata 1322A interface. Data acquisition and analysis was carried out using P Clamp software (pClamp 9 software; Axon instrument, USA). When analysing, recordings were filtered using Butterworth filters (Clampfit).

4.2.3.1 Technical challenges with two electrode voltage clamp

The primary challenge of the TEVC technique is to achieve effective voltage control, minimising voltage escape to less than 5 – 10% of the unclamped EPP response (fig 4.2). At room temperatures (18 - 20°C), I was able to achieve fairly reliable voltage clamp according to this criterion. However, when the bath temperature exceeded more than about 20°C, for example during the summer months, I experienced several difficulties. Those included generation of substantially larger endplate currents with control conditions (Hepes MPS) compared to cooler days (18 - 20°C) (Stiles et al., 1999). As a consequence of the large endplate currents, achieving adequate voltage control became quite challenging. Base line noise level also became considerably higher with the increase gain settings required to voltage clamp amplifier (fig 4.3). Therefore in some experiments, I cooled the bathing medium to less than 20°C. Mostly, I attempted to maintain good voltage control by adjusting the feedback gain without adding any oscillations to recordings.

4.2.3.2 Sampling protocol for two electrode voltage clamp measurements

Baseline recording of EPCs and MEPCs were made from 3 – 5 muscle fibres (μ-CTX pre-treated intercostal nerve / trangularis sterni nerve-muscle preparations) using the following protocol. First, MEPCs were recorded for 60sec. Then from the same fibre, EPCs at different frequencies (1Hz, 10Hz, 20Hz, 30Hz, 50Hz and 100Hz) were recorded. Then drugs were

added to the bath to achieve the targeted drug concentration. Further recordings from 10 – 15 fibres were then carried out using the same protocol as above.

4.2.4 Quantal analysis

4.2.4.1 EPP responses

In addition to the EPC analysis, I re examined EPP records I made from FDB. Quantal content measurements of were made using WinWCP software. Trains of 30 EPP recordings from each fibre were used for calculating quantal content using Variance method. Estimates from Direct method of quantal analysis (where it was possible to estimate the mean MEPPs amplitude) were used to compare the variance method values.

EPP recordings are subject to non-linear summation when the quantal content is relatively high (McLachlan and Martin, 1981). The McLachlan - Martin equation corrects EPP amplitude using the equation V' = V/ (1-f.V/E) equation (V' = corrected amplitude, V= recorded amplitude, f="fudge" (correction) factor, E = difference between the resting membrane potential and the reversal potential for the action of ACh at its receptor).

The correction is incorporated in to WinWCP and I applied it with a reversal potential of -10mV and f factor of 0.3. This gave a good empirical agreement with the direct, variance and failures method when quantal content was reduced in MPS contained low Ca^{2+} / high Mg^{2+} (R Brown PhD thesis).

4.2.4.2 EPC responses

I also measured quantal content from EPC recordings in TS preparations using the Direct Method of quantal analysis. Both amplitude and the area under curve (total charge) of spontaneous MEPC and evoked EPC analysed using Clampfit (pClamp software 9, Axon instrument, USA). If the decay time constant (τ) of MEPC recording differed from EPC recordings, then total charge was utilised. Otherwise, the ratio of the amplitude measurements was used to determine the quantal content. Endplate currents are not subjected to non-linear summation due to constant membrane voltage; therefore correction of EPC current amplitude was not required.

4.2.5 Focal extracellular recording / nerve terminal currents

Preliminary nerve terminal current recordings were obtained from TS nerve muscle preparation during a visit to Rutgers New Jersey Medical School, USA with the help of Dr. V. Patel and Prof. J. McArdle. Focal macropatch electrodes were pulled and back filled with Hepes MPS. The preparation was pre-treated with TRITC-α-bungarotoxin (30nM) to block muscle contraction and facilitate identification of the endplate under a fluorescence microscope (Patel et al., 2014). The patch electrode was manipulated above the identified NMJ and trains of 100 stimuli delivered at 1Hz using via stimulation of the intercostal nerve. Nerve terminal currents were recorded via Axoclamp 2B amplifier and analysed using pClamp software.

Fig 4.1: Sample traces showing different voltage responses to current injection. A: inactivation of inward rectifying potassium channels produces substantial amount of hyperpolarization (t_2) resulting in a non-linear current – voltage relationship (T1 and T2 are two sampling points for current – voltage relationship), B; graph showing the current-voltage relationship for the traces in A at different time points, i.e, T1 & T2. C; Poor current passing electrode characteristics resulted in sluggish action potentials with large step currents, with poor demarcation of threshold and action potential. In Traces A & C, lower panel indicate the injected current, while upper panel indicate the resulting voltage.

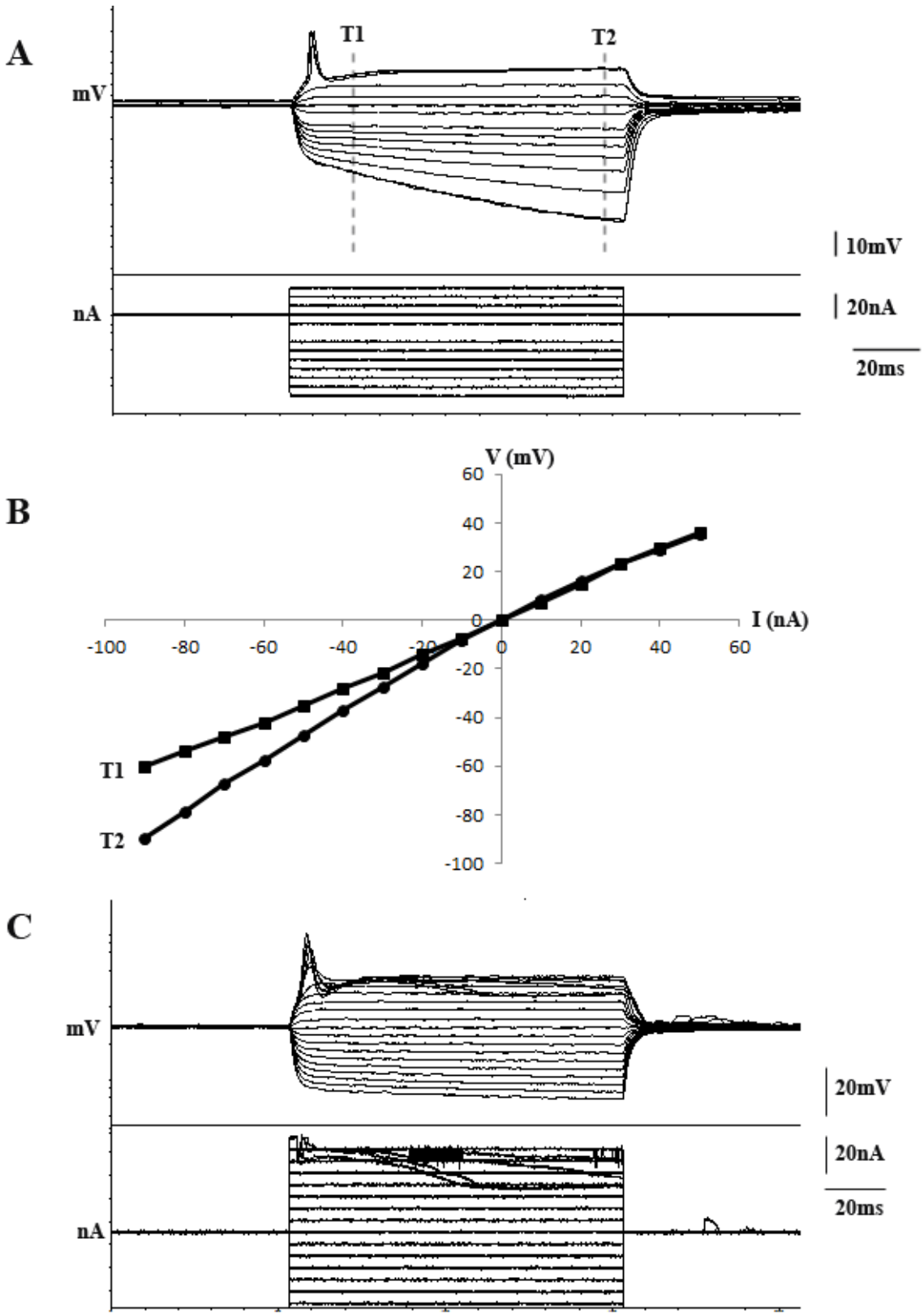
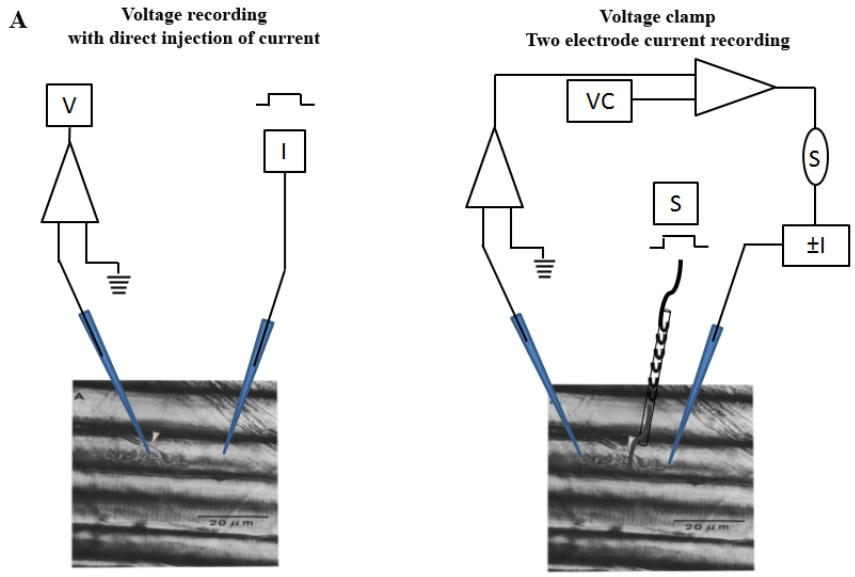
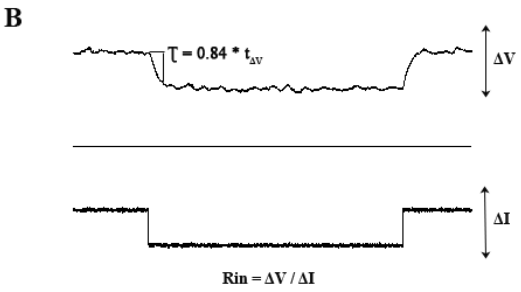

A
T1
T2
mV
nA
10mV
20nA
20ms
B
V (mV)
I (nA)
T1
T2
C
mV
nA
20mV
20nA
20ms

Fig 4.2: Voltage clamp circuitry and characteristics of the endplate currents. A: Diagrammatic illustration of the differences in circuitry in recording of voltage response to direct injection of current and two electrode current recording (Two electrode voltage clamp) (S indicate the stimulation), B; Voltage response to a step current injection (lower traces represent the injected current and upper traces represent the resulting voltage) C; analysis of the endplate currents (Top trace demonstrate the voltage escape and bottom trace demonstrate the current. The time of the stimulation was displayed by the stimulus artefact appear at the beginning of the both traces. The measured parameters of the current include; peak amplitude, latency (time between the stimulus and the beginning of the response) and rise time. Decay time was analysed as Tau (Ƭ), i.e. the time for the EPC amplitude to decay 37% of its original value or T90, time for the EPC amplitude to decay 90% of its original value.


A
Voltage recording
with direct injection of current
V
I
20 µm
Voltage clamp
Two electrode current recording
VC
S
S
±I
20 µm


B
τ = 0.84 * t_ΔV
ΔV
ΔI
Rin = ΔV / ΔI

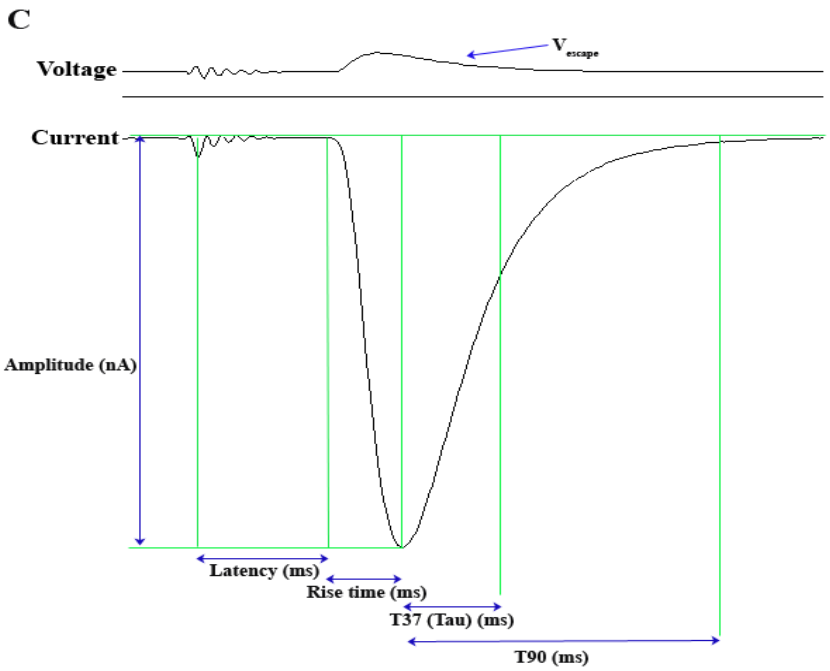
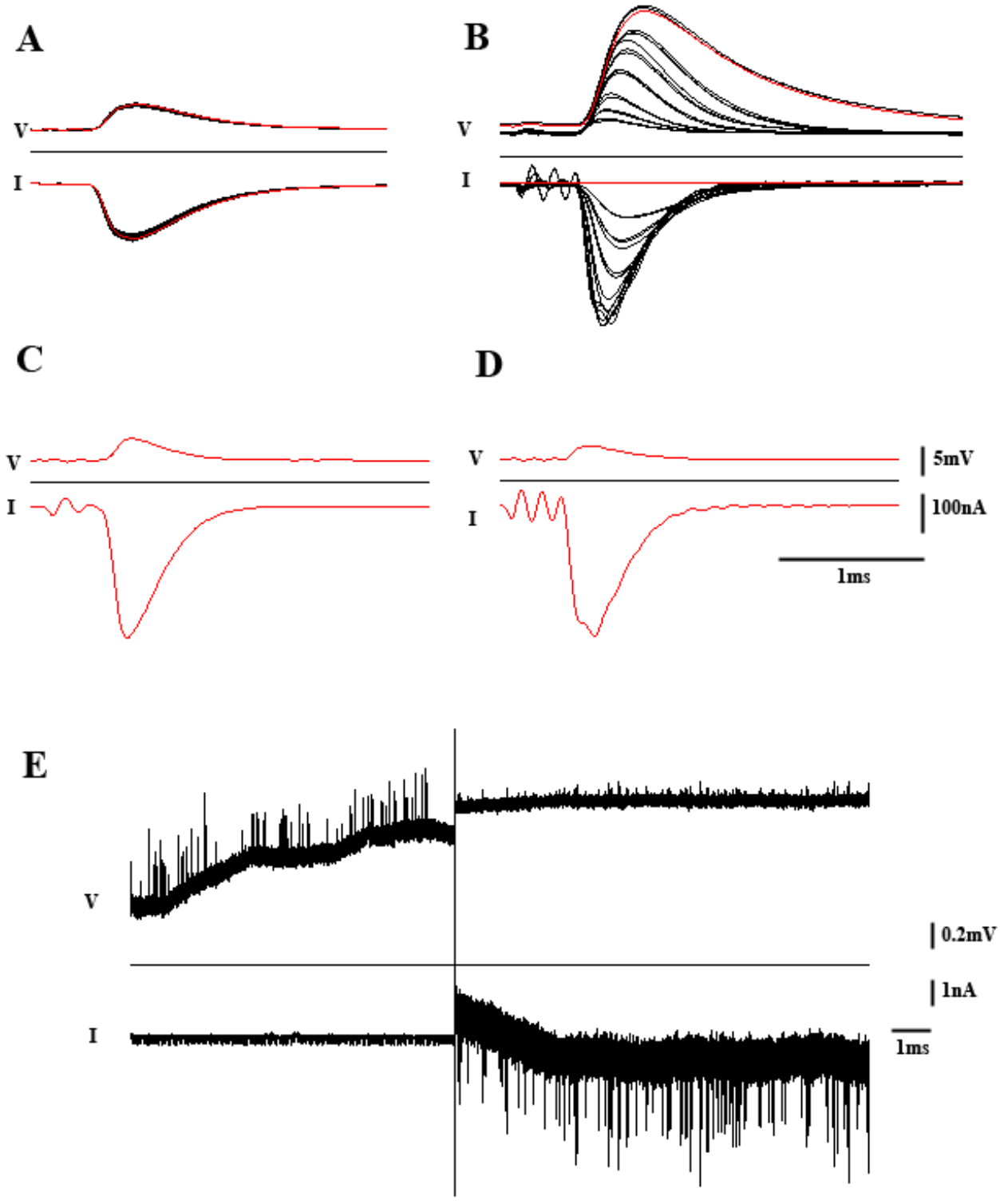

C
Voltage
V_escape
Current
Amplitude (nA)
Latency (ms)
Rise time (ms)
T37 (Tau) (ms)
T90 (ms)

Fig 4.3: Effectiveness of two electrode voltage clamp. A: TECV trace at 18°C (voltage escape is less than 10% from the original value), B; TECV trace at 25°C (note that the substantial production of current compared to trace A. Superimposed traces in B indicate the clamping of the voltage with different gain settings. Note the unclamp mode (current clamp) with red colour active trace and the amount of reduction in voltage escape with higher gain settings in voltage clamp mode. C; TECV trace at 25°C (voltage clamp is 10 – 15 % from the original value), D; TECV trace at 25°C (voltage clamp is less than 10 % from the original value – but note the oscillation of the currents caused by a high gain settings of the feedback amplifier. It was not possible using the phase lag control on the Axoclamp B amplifier to compensate for this without further loss of voltage control), E; effective voltage clamp recording MEPC at 18°C. Note the two vertical panels indicate the switching between current clamp and voltage clamp mode. Scale bars on trace D represent all the traces from A – D.


A
V
I
B
V
I
C
V
I
D
V
I
5mV
100nA
1ms
E
V
I
0.2mV
1nA
1ms

4.3 Results

4.3.1 Neither pesticide nor metabolic breakdown products changes the passive membrane properties of the muscle fibre.

The primary objective of this set of experiments was to examine whether any of the pesticide ingredients or their metabolites either singly or in combination alters the passive membrane properties of the muscle fibres. Change in passive membrane properties would affect the magnitude of the endplate potential and would also change the time course of the response of membrane potential after DC current injection.

The data revealed that neither pesticide nor their metabolites change the input resistance of the muscle fibre (MPS: 0.31±0.09MΩ, dimethoate; 0.23±0.04 MΩ, cyclohexanone; 0.33±0.09 MΩ, omethoate; 0.30±0.11 MΩ, cyclohexanol; 0.29±0.05 MΩ, omethoate + cyclohexanol; 0.32±0.09 MΩ, DOCC; 0.29±0.06 MΩ, mean ± SEM, $P > 0.05$, ANOVA, "$F = 0.5$", $n = 3$ muscles per each treatment group) (fig 4.4). Assuming muscle fibre diameter was relatively constant for all the experiments, I concluded that none of these treatments changed the specific membrane resistance (i.e. the general leakiness of the muscle fibre membrane).

Next, I measured the time constant of the voltage response by calculating the 84% of the time taken to produce steady state voltage response after injecting step current. As discussed in Chapter 1, membrane time constant is a product of input resistance and input capacitance of the muscle fibres. This determines the time it takes membrane potential to change in response to a step injection of current. The results showed that none of the treatments altered the time constant. Taken together with the Rin measurements, this supports that there were no effect of the components on the membrane capacitance either (τ) MPS: 4.79±0.64ms, dimethoate; 5.21±0.56 ms, cyclohexanone; 4.45±0.77 ms, omethoate; 5.09±0.96 ms, cyclohexanol; 4.48±0.57 ms, omethoate + cyclohexanol; 4.25±0.54 ms, DOCC; 4.96±0.55 ms, mean ± SEM, $P > 0.05$, ANOVA, "$F = 1.33$") (fig 4.5).

Next I examined the action potential threshold for each of the treatments. I wanted to establish whether these treatments had any direct effects on action potential initiation in the muscle fibres. If any of the treatments elevated the action potential threshold, then the probability of producing muscle action potential would become reduced, perhaps thereby contributing to overall failure of neuromuscular transmission. However, the data did not reveal any differences in action potential threshold with any treatments (MPS: -

52.19±2.32mV, dimethoate; -53.23±2.11 mV, cyclohexanone; -51.24±1.69 mV, omethoate; -51.36±1.87 mV, cyclohexanol; -49.69±5.18 mV, omethoate + cyclohexanol; -51.14±2.53 mV, DOCC; -50.96±1.36 mV, mean ± SEM, P > 0.05, ANOVA, "F= 0.9") (fig 4.6B). I also measured the currents required to produce an action potential with these treatments. Similar to measurements of the action potential threshold, none of the treatments treatments produced any changes in the magnitude of the current required to produce an action potential (MPS: 51.70±8.64nA, dimethoate; 65.88±3.11 nA, cyclohexanone; 53.85±5.67 nA, omethoate; 55.58±16.72 nA, cyclohexanol; 51.79±10.85 nA, omethoate + cyclohexanol; 51.25±18.49 nA, DOCC; 61.02±12.65 mV, mean ± SEM, P > 0.05, ANOVA, "F = 0.1") (fig 4.6C).

Therefore, from the data obtained it appears that pesticide components and their metabolites do not alter either the passive membrane properties or the voltage gated ion channels that underlay muscle fibre excitability.

Fig 4.4: Input resistance of the muscle fibre. A: traces displaying production of voltage differences as a result of direct step currents injected to muscle fibre, B; current – voltage relationship in response to hyperpolarizing current injected (input resistance was measured as a slope function of current – voltage plot), Each point represent the voltage response to a step current in control condition. C; comparison of input resistance measurements under different treatments. Each bar represents mean ± SEM (n = 3 muscles per each treatment group) ($P > 0.05$, **ANOVA,**)

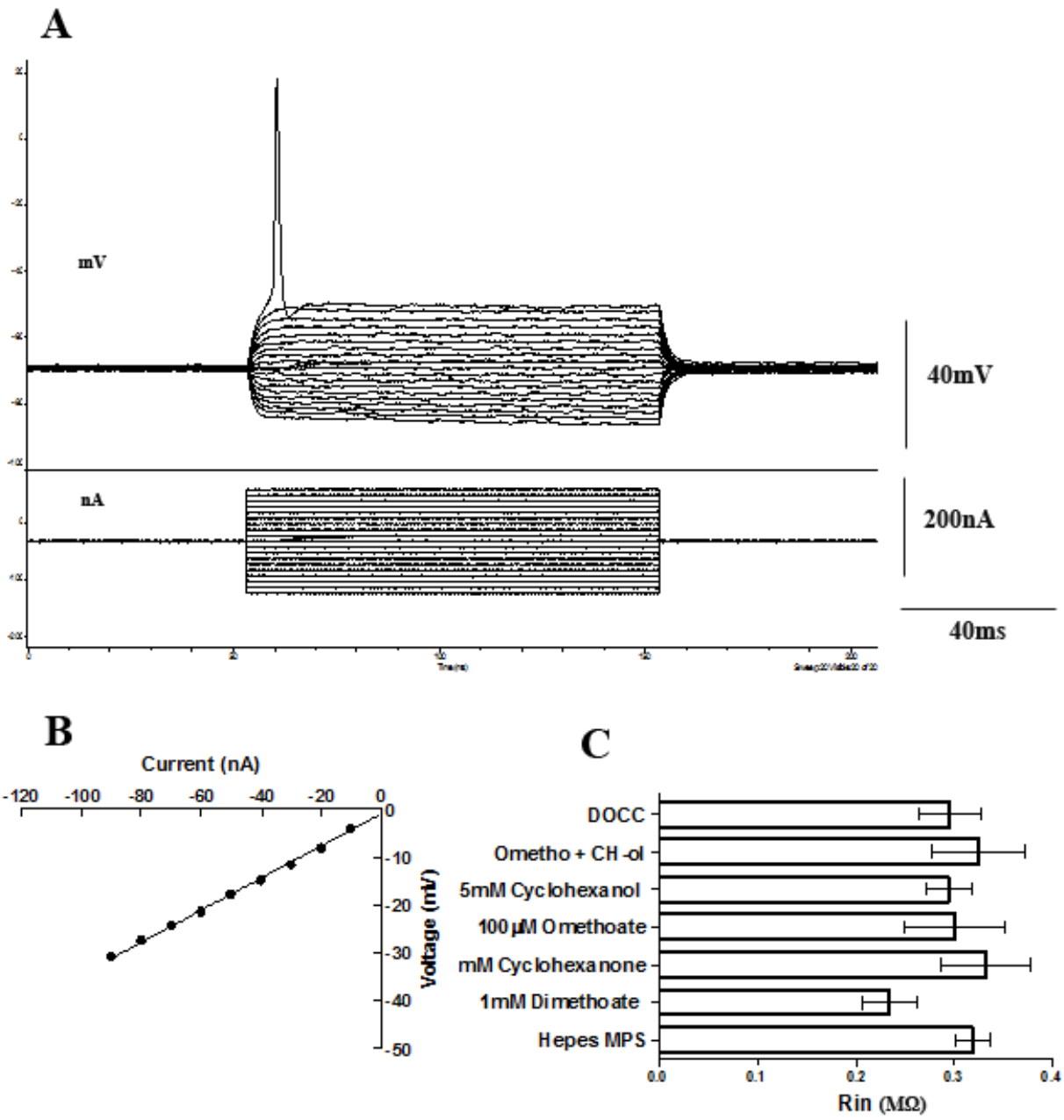
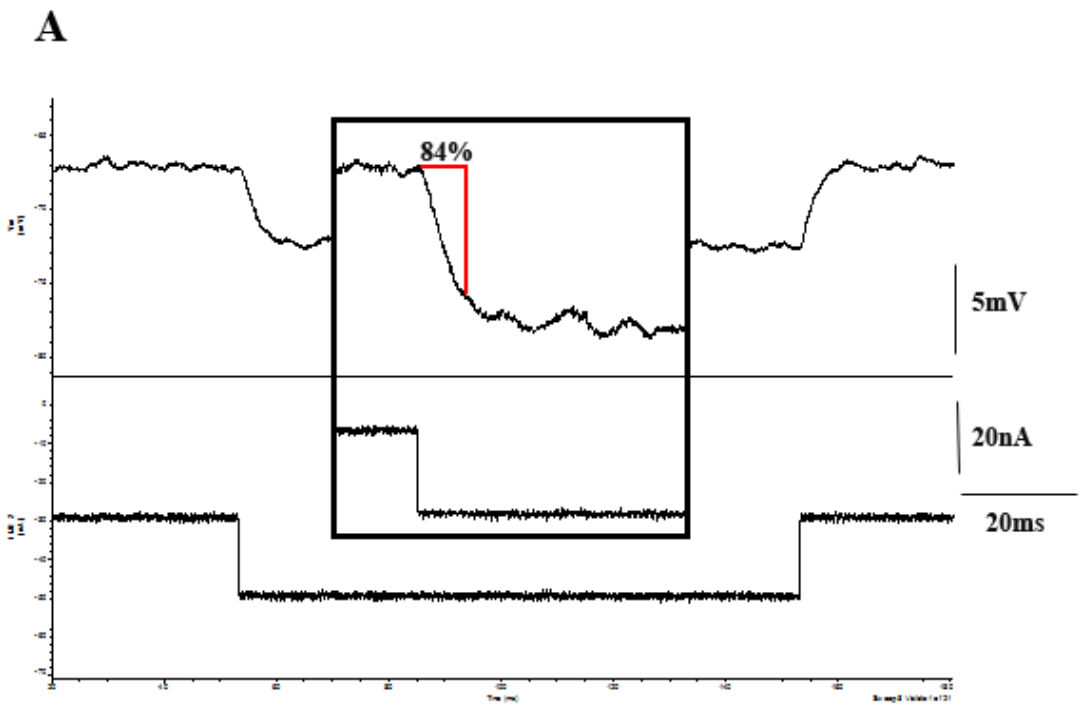

A
mV
nA
40mV
200nA
40ms
B
Current (nA)
-120
-100
-80
-60
-40
-20
0
-10
-20
-30
-40
-50
Voltage (mV)
C
DOCC
Ometho + CH -ol
5mM Cyclohexanol
100 µM Omethoate
mM Cyclohexanone
1mM Dimethoate
Hepes MPS
0.0
0.1
0.2
0.3
0.4
Rin (MΩ)

Fig 4.5: Membrane time constant (Ƭ) with different treatments. A: measurement of membrane time constant with using voltage response produced to constant current (note that the 84% of the ΔV_m represented by Ƭ), B; bar chart showing differences in Ƭ with pesticide components and its metabolites. Each bar represents mean ± SEM (n = 5 muscles per each treatment group) ($P > 0.05$, **ANOVA**)


A
84%
5mV
20nA
20ms

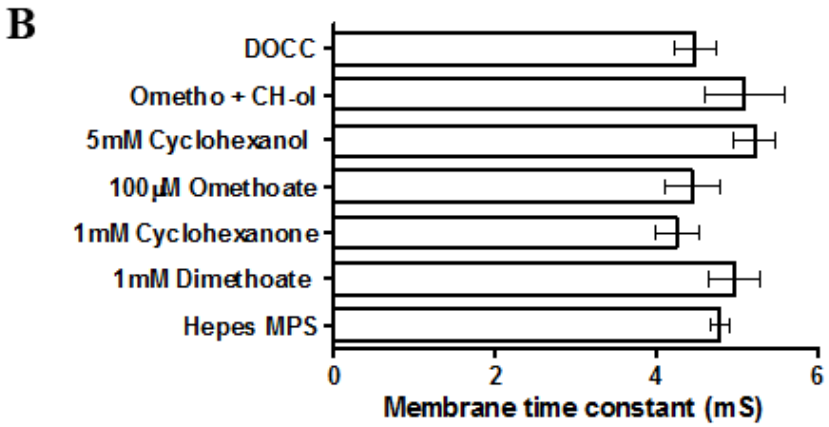
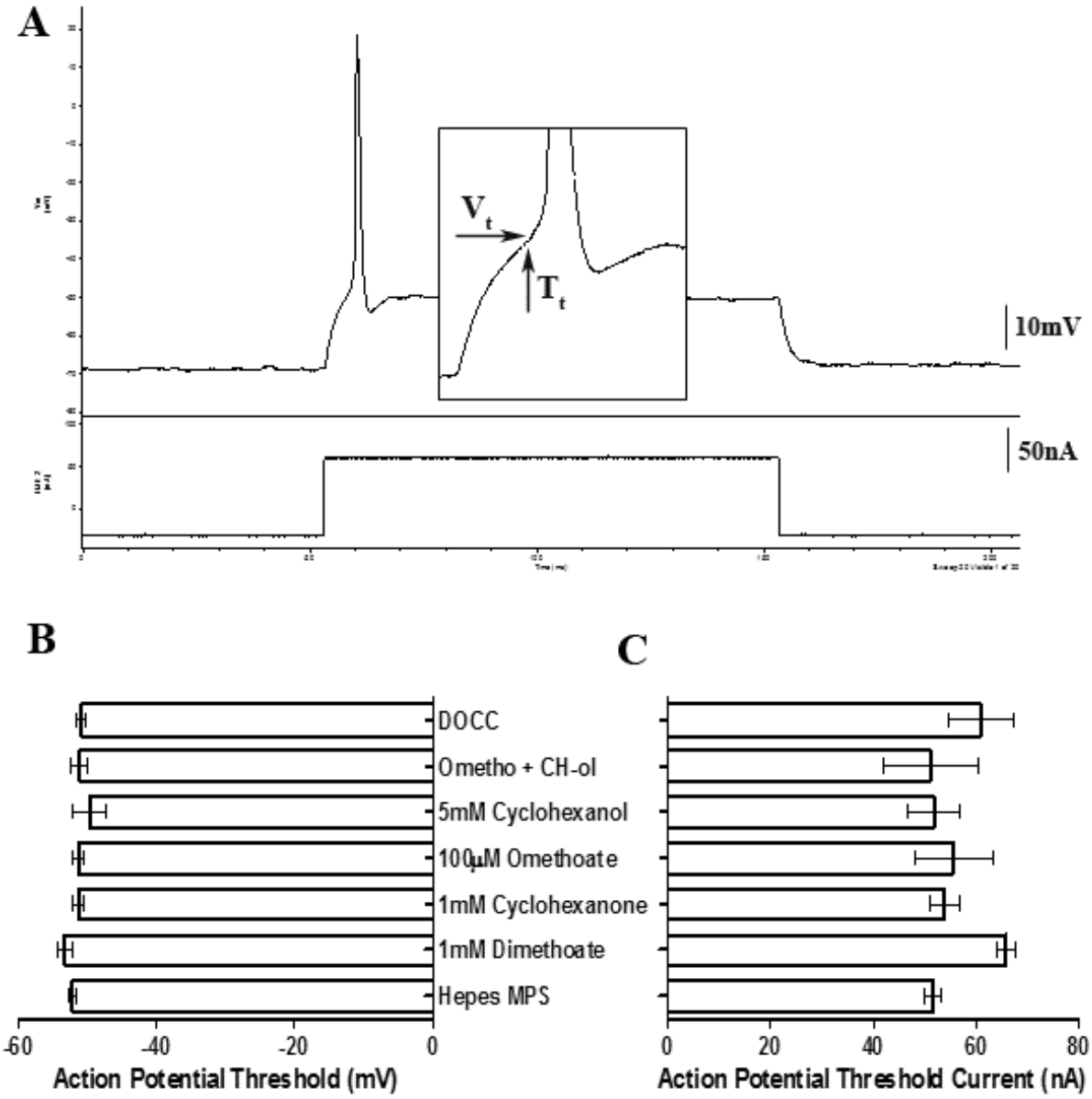

B
DOCC
Ometho + CH-ol
5mM Cyclohexanol
100µM Omethoate
1mM Cyclohexanone
1mM Dimethoate
Hepes MPS
0
2
4
6
Membrane time constant (mS)

Fig 4.6: Comparison of the action potential threshold and currents with different treatments. A: Traces showing generation of action potential and amount of current required (highlighted area is to display the action potential threshold level), B; different threshold values of action potential with pesticide components and its metabolites, C; the current requirement to produce action potentials with different treatments. V_t and T_t are the voltage and time at the inflexion points decides the threshold of the action potential (see page 153). Each bar represent mean ± SEM (n = 3 muscles per each treatment group) ($p > 0.05$, **ANOVA**)


A
V_t
T_t
10mV
50nA
B
C
DOCC
Ometho + CH-ol
5mM Cyclohexanol
100µM Omethoate
1mM Cyclohexanone
1mM Dimethoate
Hepes MPS
-60
-40
-20
0
Action Potential Threshold (mV)
0
20
40
60
80
Action Potential Threshold Current (nA)

4.3.2 Omethoate prolonged EPCs

In this set of experiments I aimed to understand how omethoate and cyclohexanol, either singly or in combination, act on synaptic currents.

Data from TEVC recordings showed that omethoate produced significant prolongation of synaptic current decay time for both MEPC and EPC (Fig 4.7, 4.8, 4.9). Surprisingly, however cyclohexanol did not increase the decay time constant (MEPC - Hepes MPS: 0.95±0.18ms, omethoate; 2.48±0.95ms, cyclohexanol: 0.68±0.40ms, omethoate + cyclohexanol; 1.94±0.25ms, mean ± SEM, $P < 0.05$, ANOVA, "F15.76") (Fig 4.7, 4.8, 4.9). Peak amplitudes of the treated preparations were not significantly different compared to control (Hepes MPS: -2.47±0.74nA, omethoate; -2.50±0.39nA, cyclohexanol: -2.43±0.30nA, omethoate + cyclohexanol; -2.19±0.87nA, mean ± SEM, $P > 0.05$, ANOVA, "F = 0.02") (fig 4.9).

Analysis of the EPC showed no difference in peak amplitude with treatments either singly or in combination (Hepes MPS: -268.06±127.18nA, omethoate; -247.13±70.73nA, cyclohexanol: -320.24±58.74nA, omethoate + cyclohexanol; -273.42±98.41nA, mean ± SEM, $P > 0.05$, ANOVA, "F = 0.3") (fig 4.10). However, the half decay time of the EPCs with different treatments showed significant prolongation with omethoate and combination of omethoate and cyclohexanol treated preparations (Hepes MPS: 0.69±0.07mS, omethoate; 6.38±72.35mS, cyclohexanol: 0.83±0.05mS, omethoate + cyclohexanol; 4.53±0.78mS, mean ± SEM, $P < 0.05$, ANOVA, "F = 18.45")(fig 4.10). However the normalized area (unit integrative charge) measurements showed different patern of increment compared to half decay time increament. This is also showen in Fig. 4.11 comparing consecutive traces of single experiment for each treatment, demonstrating different decay kinetics in these preparations. Interestingly, when I attempted to fit exponential curves to the EPCs recordings, those in Hepes MPS showed single exponential decay, consistent with classical AChR kinetics (Kordas, 1972). However, both omethoate and cyclohexanol treated preparations required double exponential fits to their EPC decay time. Moreover, the combination of omethoate and cyclohexanol treated preparation EPCs sometimes required more than two exponentials to fits to their decay time, suggesting decay time function of those treatments together are complex and independent from one another (fig 4.12).

The last EPC in response to train of high frequency (50Hz) repetitive stimulation revealed a significant prolongation of the decay time with combination of omethoate and cyclohexanol compared to Hepes only trains (fig 4.13). This observation demonstrates the more

pronounced effects of combination of omethoate and cyclohexanol in repetitive high frequency burst stimuli compared to low frequency, single stimuli.

Fig 4.7: Comparison of MEPC traces with different treatments. A-a: Hepes MPS, B-b; cyclohexanol (5mM), C-c; omethoate (100µM), D-d; omethoate and cyclohexanol. A – D traces represent the MEPC over 10s time period while a – d represent single MEPC in each treatment. Note that the decrease MEPC amplitude in cyclohexanol treated preparations while, increase decay time in omethoate treated preparations. (Average MEPC trace was shown in the boxes). A – D traces represent a slower time base and show the distribution of MEPC, while a – d traces represent a faster time base and demonstrate characteristics of single MEPC.

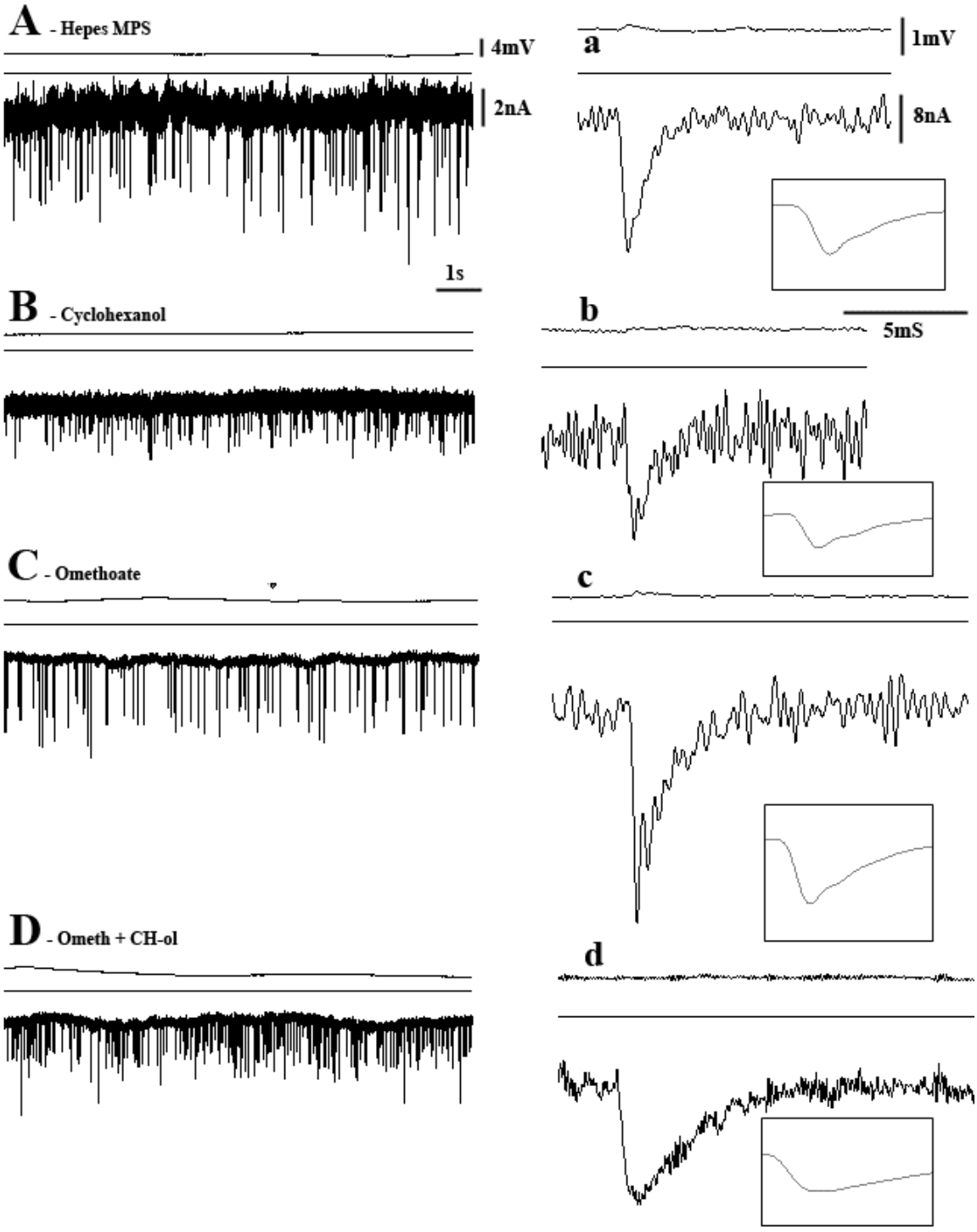

A - Hepes MPS
4mV
2nA
1s
a
1mV
8nA
b
5mS
B - Cyclohexanol
c
C - Omethoate
d
D - Ometh + CH-ol

Fig 4.8: Comparison of the example EPC traces with different treatments. A: Hepes MPS, B; omethoate (Note the increase yet complex decay phase in omethoate treated preparations), C; cyclohexanol, D; omethoate and cyclohexanol (interestingly, combination of two metabolites produced more prolonged decay phase than that of omethoate alone. However cyclohexanol alone did not prolong the decay phase, suggesting more complex effects of those two components together on post synaptic nAChRs).

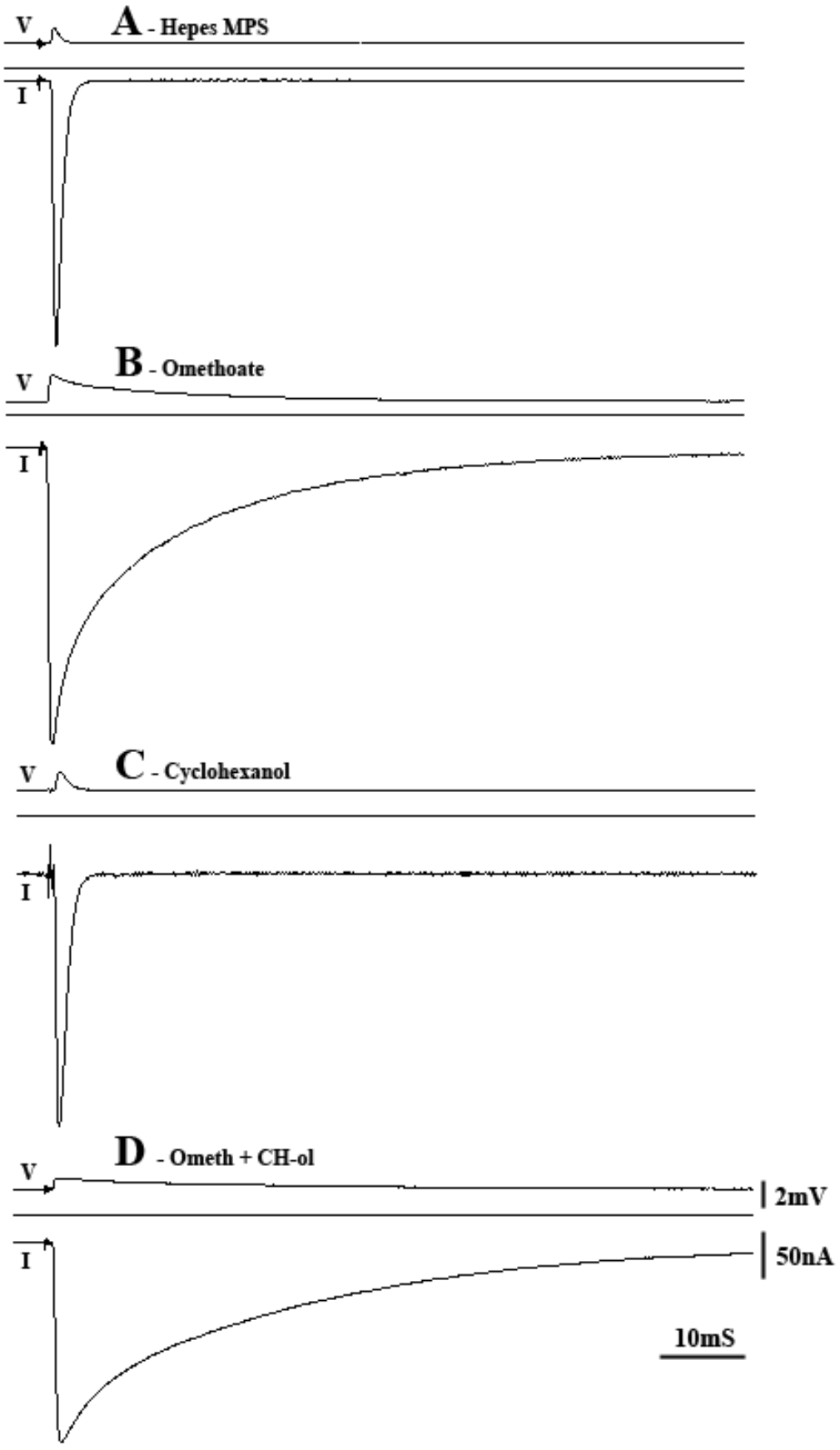
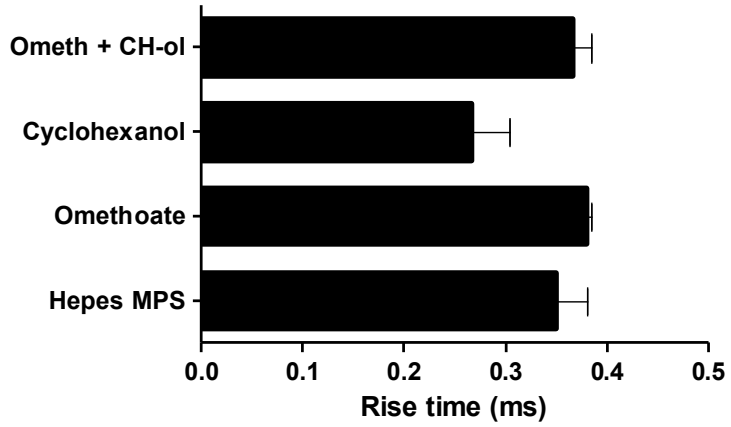
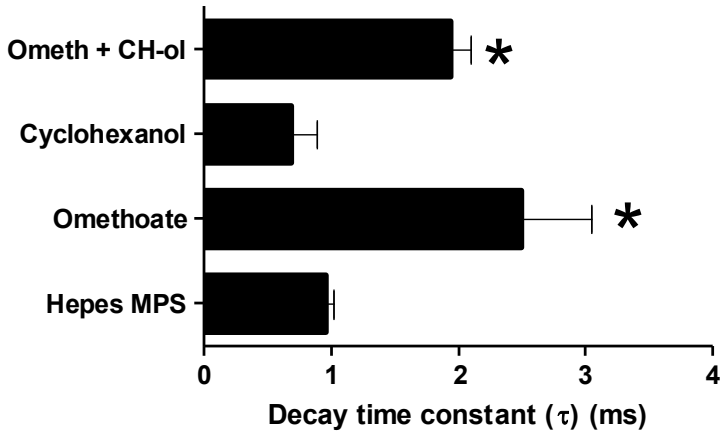

V
A - Hepes MPS
I
B - Omethoate
V
I
C - Cyclohexanol
V
I
D - Ometh + CH-ol
V
2mV
I
50nA
10mS

Fig 4.9: Quantitative analysis of MEPC parameters with different treatments. Statistically significant increase in decay time constant was observed in omethoate treated preparation. Each bar represents mean ± SEM [n (No of muscles): Hepes MPS = 11, omethoate = 3, cyclohexanol = 5, omethoate and cyclohexanol = 3] ($p < 0.05$, **ANOVA, Bonferroni post test-treatements were compared with Hepes MPS**)


Ometh + CH-ol
Cyclohexanol
Omethoate
Hepes MPS
0.0
0.1
0.2
0.3
0.4
0.5
Rise time (ms)


Ometh + CH-ol
*
Cyclohexanol
Omethoate
*
Hepes MPS
0
1
2
3
4
Decay time constant (τ) (ms)

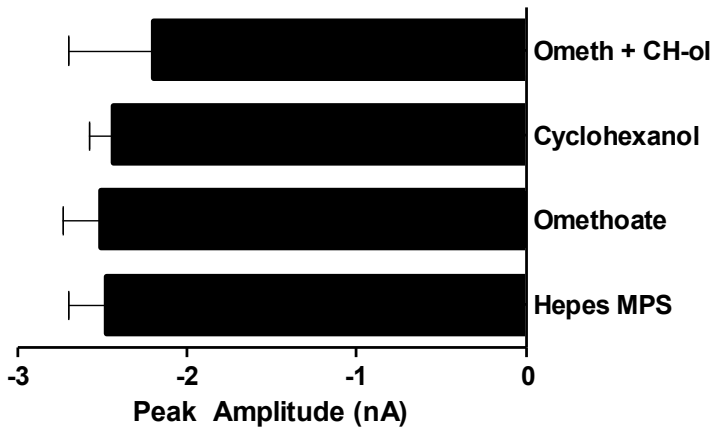
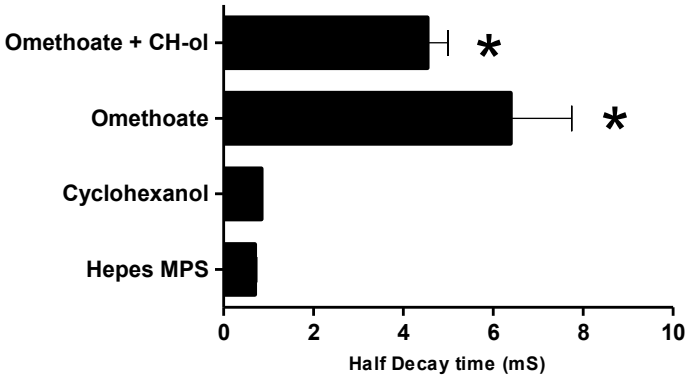
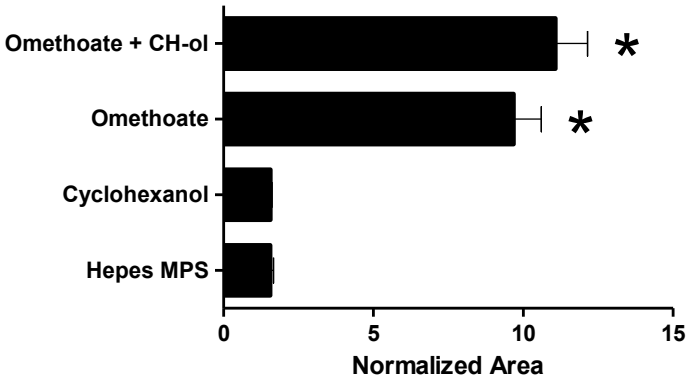

Ometh + CH-ol
Cyclohexanol
Omethoate
Hepes MPS
-3
-2
-1
0
Peak Amplitude (nA)

Fig 4.10: Qualitative analysis of EPC parameters with different treatments. Similar to MEPC, omethoate treated preparation shows an increase in half decay time. However, comparison of normalized area (unit integrative charge) (area/peak amplitude) measurements showed different pattern of increase compared to half decay time increment. Each bar represents mean ± SEM [n (No of muscles): Hepes MPS = 11, omethoate = 3, cyclohexanol = 5, omethoate and cyclohexanol = 3] ($p < 0.05$, **ANOVA, Bonferroni post test- treatements were compared with Hepes MPS**)


Omethoate + CH-ol
Omethoate
Cyclohexanol
Hepes MPS
*
*
0
2
4
6
8
10
Half Decay time (mS)


Omethoate + CH-ol
Omethoate
Cyclohexanol
Hepes MPS
*
*
0
5
10
15
Normalized Area

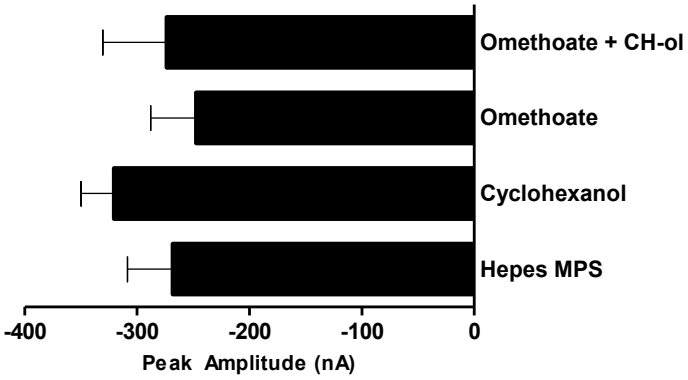

Omethoate + CH-ol
Omethoate
Cyclohexanol
Hepes MPS
-400
-300
-200
-100
0
Peak Amplitude (nA)

Fig 4.11: Traces (EPC) comparing time course of the treatments (10 consecutive traces from single experiment for each treatment). Sampling of EPCs were carried in every 8 – 10 minutes. A: EPCs of cyclohexanol treated preparations, B; EPCs of omethoate treated preparations, C; EPCs of cyclohexanol and omethoate treated preparations. Downward arrow indicates the progression of time. Note that the cyclohexanol treatment does not increase the decay time of EPC, in the contrary omethoate treatement increase the time course substancially. There is also some evidence of desensitization in omethoate treated preparation with the progression of time.

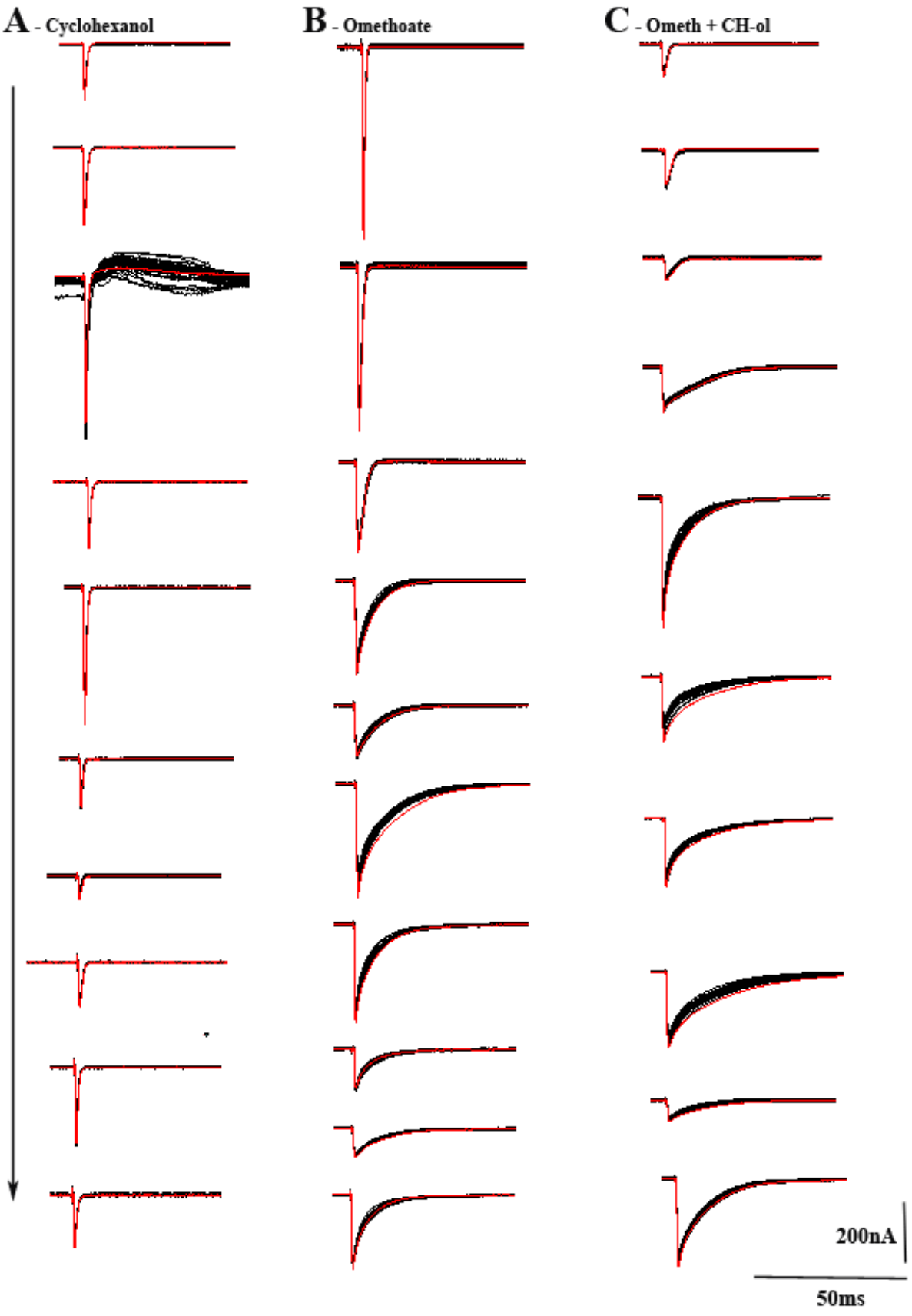

A - Cyclohexanol
B - Omethoate
C - Ometh + CH-ol
200nA
50ms

Fig 4.12: Exponential decay time fit with different treatments. A: Hepes MPS, B; omethoate, C; cyclohexanol, D; omethoate and cyclohexanol. Traces in B and D panels clearly demonstrate the multiple exponential fit to decay time. Trace C also shows slight deviation from the single exponential decay. N_e = number of exponential components.

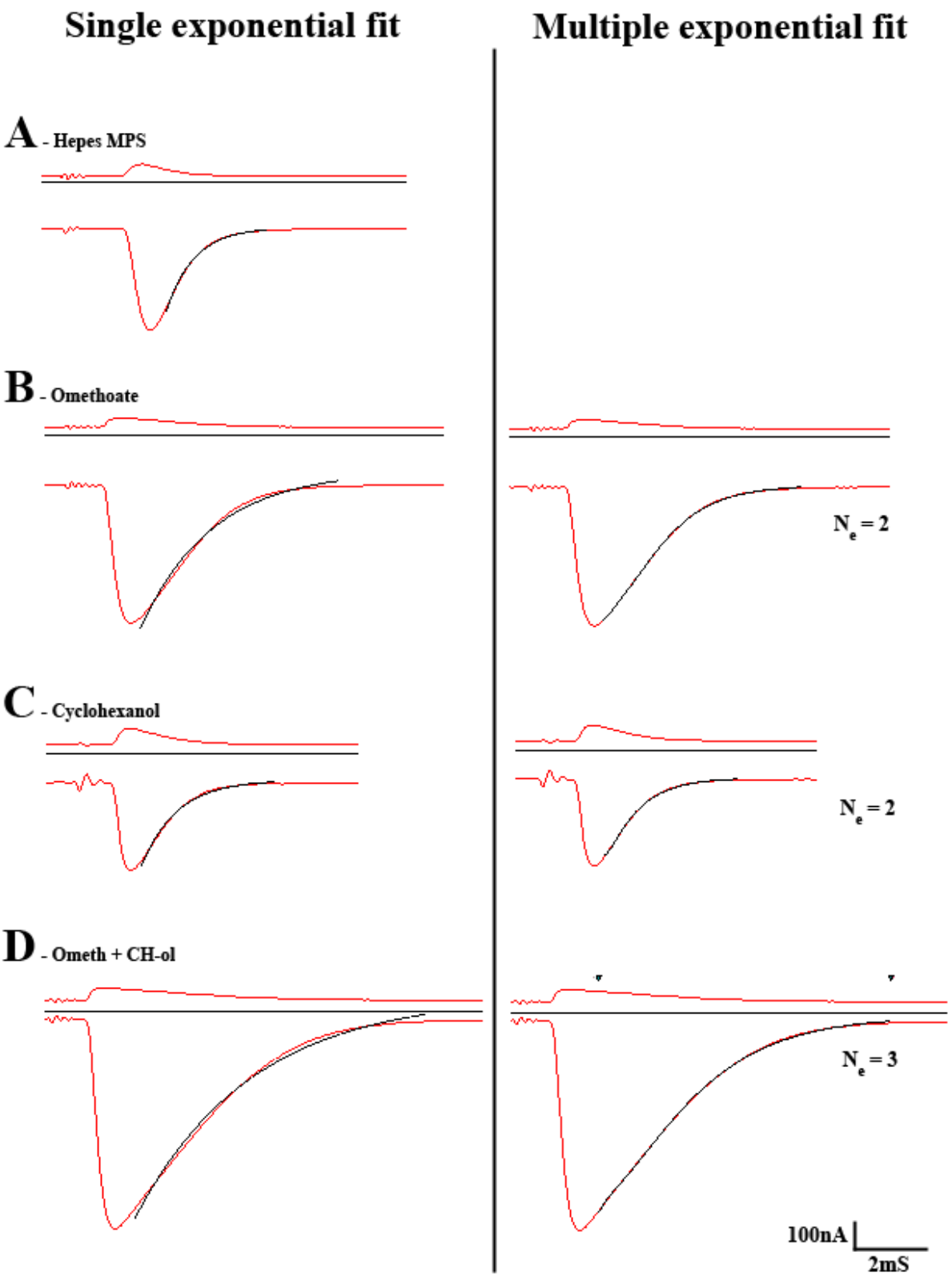
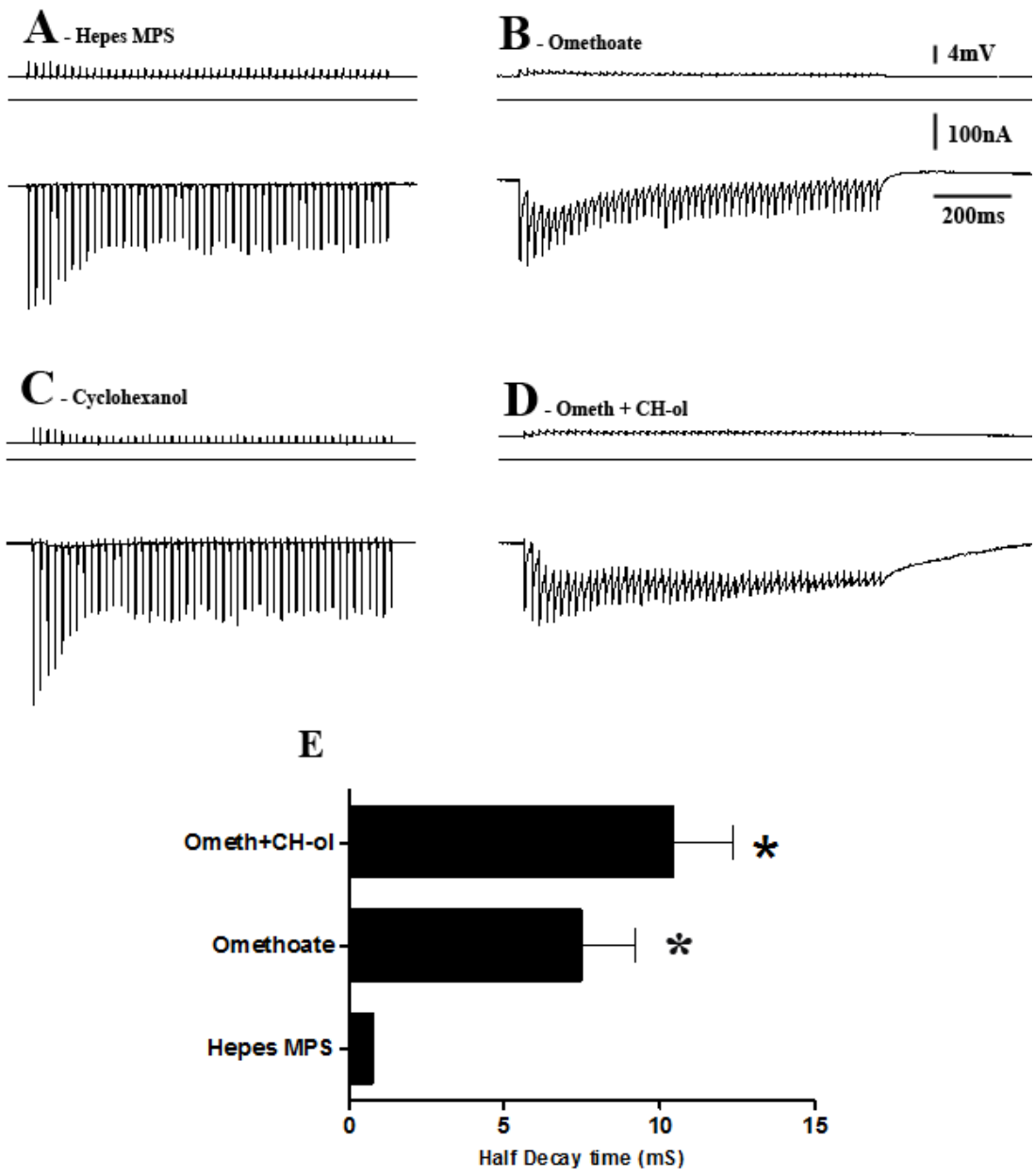

Single exponential fit
Multiple exponential fit
A - Hepes MPS
B - Omethoate
$N_e = 2$
C - Cyclohexanol
$N_e = 2$
D - Ometh + CH-ol
$N_e = 3$
100nA
2mS

Fig 4.13: Decay time of last EPC on 50Hz repetitive stimulation. A: Hepes MPS traces, B; omethoate traces, C; cyclohexanol traces, D; omethoate and cyclohexanol traces, E; qualitative analysis of half decay time of last EPC of trains of stimuli with different treatments. Note the increase in half decay time when combination of omethoate and cyclohexanol were added to the preparation. Each bar represents mean ± SEM [n (No of muscles): Hepes MPS = 11, omethoate = 3, cyclohexanol = 5, omethoate and cyclohexanol = 3] ($p < 0.05$, **ANOVA, Bonferroni post test-treatements were compared with Hepes MPS**).


A - Hepes MPS
B - Omethoate
4mV
100nA
200ms
C - Cyclohexanol
D - Ometh + CH-ol
E
Ometh+CH-ol
Omethoate
Hepes MPS
*
*
0
5
10
15
Half Decay time (mS)

4.3.3 Pesticide metabolites modulate transmitter release from the presynaptic terminal

Since I observed complex post synaptic responses with high frequency repetitive stimulation with both EPP (Chapter 3) and EPC (fig 4.13), I also asked whether there might be presynaptic effects of pesticide components and their metabolites. First I evaluated the quantal content (amount of transmitter released during evoked responses) during trains of stimuli. I initially used EPP recordings from FDB muscle fibres treated with pesticide ingredients, either singly or in combination. Unfortunately, I could not quantify the data obtained in response to 50Hz stimuli because of complex nature of the response (see Chapter 3). I therefore analysed 10Hz trains (this frequency is also close to the human respiratory muscle firing frequency). From the data (fig 4.14) I observed a slight reduction in quantal content in the presence of dimethoate, omethoate and cyclohexanol compared with Hepes MPS. However, the most surprising result was a significant reduction of quantal content when all the components of poisoned plasma were combined (Hepes MPS: 63.09±19.25 quanta, dimethoate: 25.92±7.62 quanta, omethoate: 30±7.62 quanta, cyclohexanone: 47.51±10.72 quanta, cyclohexanol: 20.86±4.25 quanta, omethoate and cyclohexanol 13.96±7.61 quanta, dimethoate + omethoate + cyclohexanone + cyclohexanol: 8.09±2.8 quanta, mean ± SEM, $P < 0.05$, ANOVA, "$F = 9.96$").

The main disadvantage of using the EPP to calculate quantal content is the effect of non-linear summation, due to the changing driving force on EPP amplitude. Despite correction for non-linear summation (McLachlan and Martin, 1981), misleading values for quantal content (both over and under estimates) may still be obtained when comparing the pharmacological effects of different drug levels. It is therefore best practice to estimate quantal content from endplate currents obtained at a fixed membrane potential, when the driving force is always constant (assuming no significant escape from voltage control). Therefore, I further analysed the quantal content in the presence of omethoate and cyclohexanol either singly or in combination using the direct method of dividing EPC amplitude by MEPC amplitude.

Under these conditions, I did not see a statistically significant change in quantal content with any of the treatments either singly or in combination (fig 4.15) MPS: 101.19±25.47 quanta, omethoate; 103.31±19.62 quanta, cyclohexanol; 125.29±13.68 quanta, omethoate and cyclohexanol; 121.42±6.01 quanta, mean ± SEM, $P > 0.05$, ANOVA, "$F = 1.64$"). Increase in sample size would perhaps resolve any type II statistical error that might underlying these data.

Fig 4.14: Comparison of quantal content estimated using EPPs with different combinations of the pesticide components in FDB preparations. CH-one, CH-ol, DOCC represent cyclohexanone, cyclohexanol and dimethoate, omethoate, cyclohexanone and cyclohexanol respectively. Each bar represents mean ± SEM [n (No of muscles): Hepes MPS = 18, dimethoate = 3, omethoate = 3, cyclohexanone = 3, cyclohexanol = 3, omethoate and cyclohexanol = 3, DOCC = 3] ($p < 0.05$, **ANOVA, Bonferroni post test-treatements were compared with Hepes MPS**). (Estimates were carried out using variance method of quantal analysis).

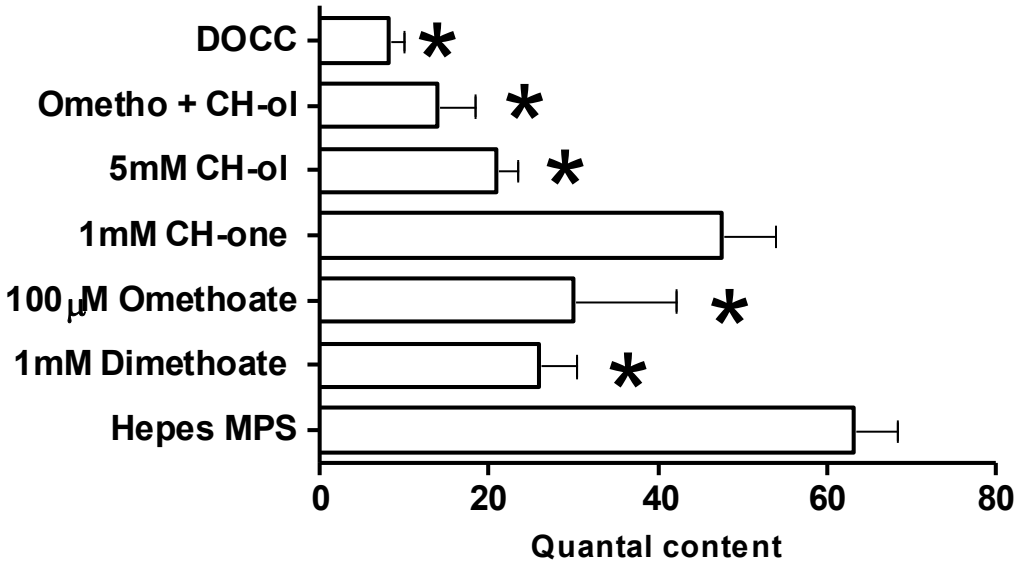
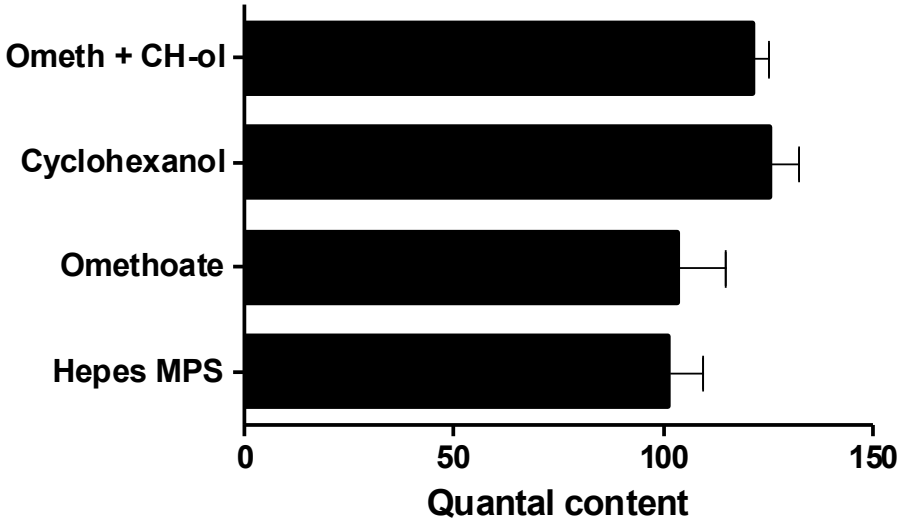

DOCC
Ometho + CH-ol
5mM CH-ol
1mM CH-one
100 μM Omethoate
1mM Dimethoate
Hepes MPS
*
*
*
*
*
0
20
40
60
80
Quantal content

Fig 4.15: Comparison of quantal content estimated using EPCs with pesticide metabolites in TS preparations. Each bar represents mean ± SEM [n (No of muscles): Hepes MPS = 11, omethoate = 3, cyclohexanol = 5, omethoate and cyclohexanol = 3] ($p > 0.05$, **ANOVA**). (Estimates were carried out using direct method of quantal content).

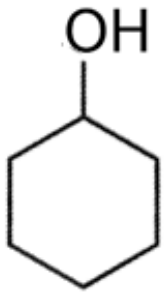

Ometh + CH-ol
Cyclohexanol
Omethoate
Hepes MPS
0
50
100
150
Quantal content

4.3.4 Cyclohexanol has complex effects on presynaptic terminals.

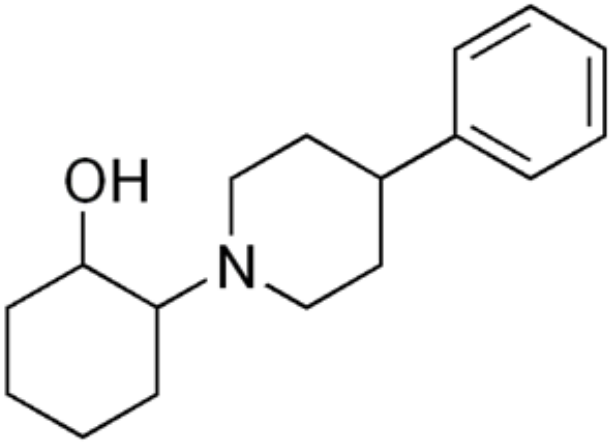
The slight reduction in MEPC amplitude along with slight increase in quantal content (EPC recordings) that I observed in the presence of cyclohexanol suggested to me that this component could be modulating transmitter release from presynaptic terminals. Therefore I examined more closely whether cyclohexanol reduces the quantal size (amount of transmitter in a single vesicle). One possible mechanism behind a reduced quantal size would be through depletion of the ACh content of synaptic vesicles, perhaps mediated by inhibition of transport into vesicles. This has previously observed in pharmacological inhibition of ACh vesicular transport using vesamicol (Prior et al., 1992), which is a derivative of cyclohexanol (Fig 4.16). In order to test this hypothesis, I recorded MEPCs in the presence of cyclohexanol immediately after indirect nerve stimulation (10Hz, for 1minute) in order to detect the stimulation effects (Searl et al., 1991). Qualitative analysis of the traces showed that reduction in MEPC amplitude with cyclohexanol after intermittent indirect stimulation (fig 4.17). This observation merits further investigations.

Finally, to test for a direct effects of cyclohexanol on presynaptic currents, I made preliminary observations using focal current recordings (these experiments were carried out in Rutgers University – Newark, USA in Prof. J.J. McArdle's lab). Qualitative data suggested there is a change in focal current waveform with cyclohexanol treated preparations compared to Hepes MPS (fig 4.18). These observations also warrant future investigation.

Fig 4.16: Comparison of the structure of cyclohexanol and vesamicol. Note that the vesamicol is a derivative of cyclohexanol, both structures contain functional hydroxyl group attached to a benzene ring.



Cyclohexanol



2-(4-phenyl-1-piperidyl)cyclohexan-1-ol (vesamicol)

Fig 4.17: Vesicular depletion protocol with 5mM cyclohexanol. Note the reduction in MEPC amplitude after each cycle of acute stimulation. (Traces are from a single experiment / muscle fibre recorded continuously over the time)

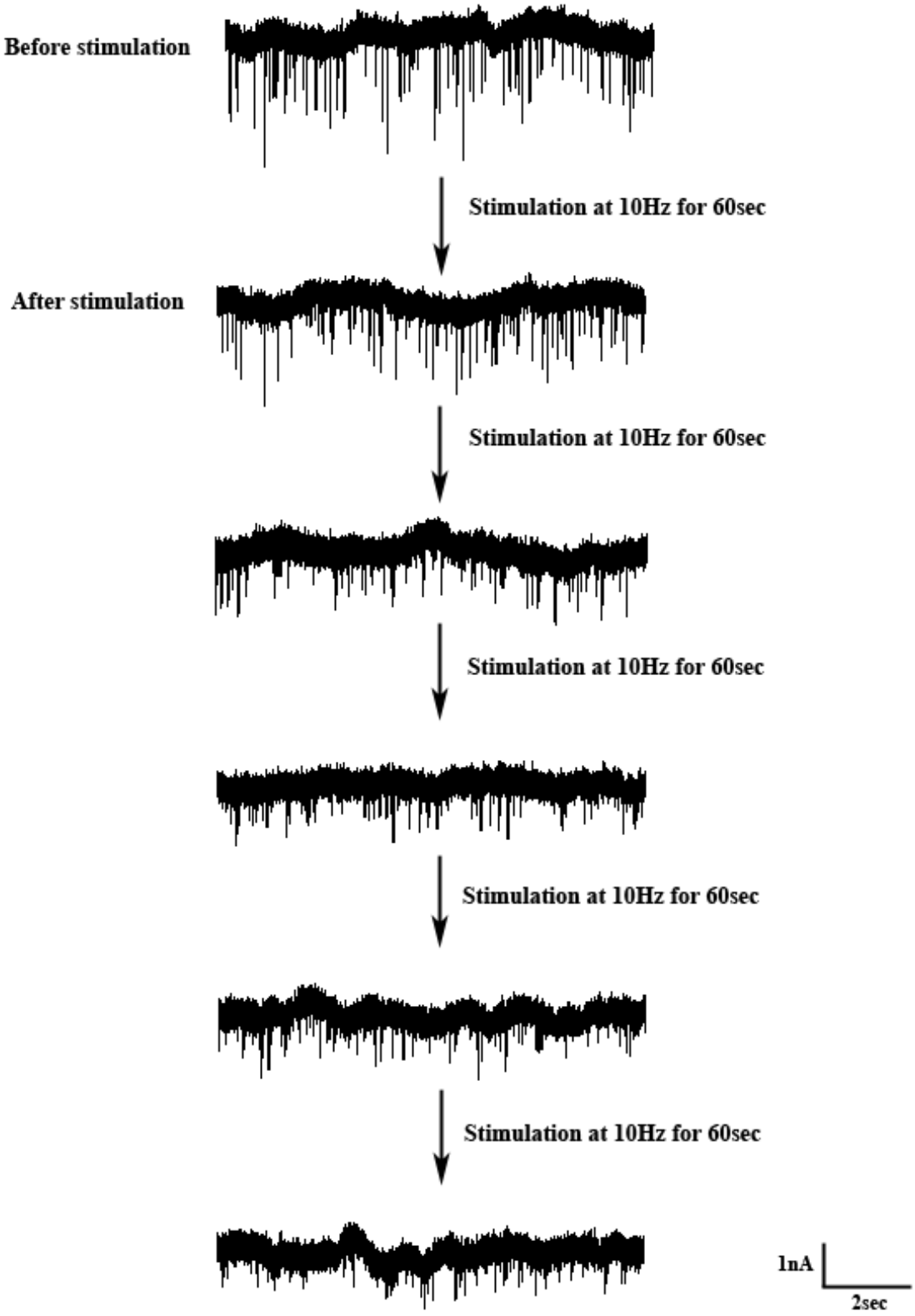
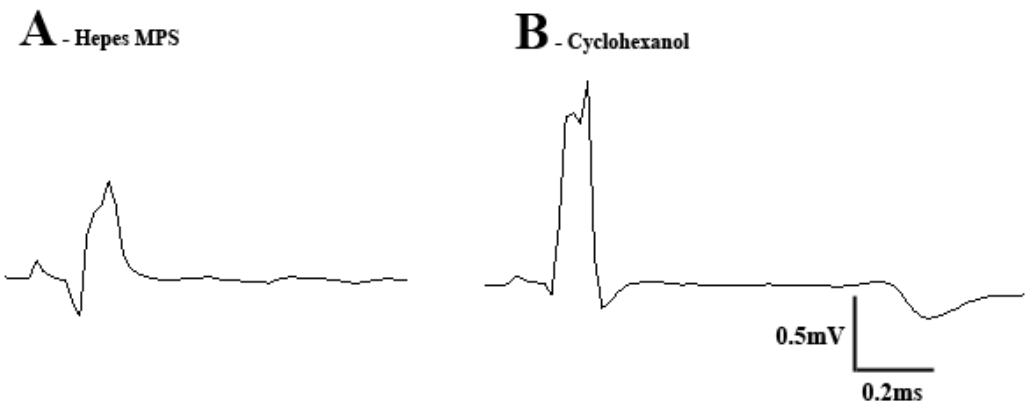

Before stimulation
Stimulation at 10Hz for 60sec
After stimulation
Stimulation at 10Hz for 60sec
Stimulation at 10Hz for 60sec
Stimulation at 10Hz for 60sec
Stimulation at 10Hz for 60sec
1nA
2sec

Fig 4.18: comparison of nerve terminal current traces with Hepes MPS and 5mM cyclohexanol treated preparations. A: Hepes MPS, B; 5mM Cyclohexanol. Note the changes in nerve terminal current waveform with cyclohexanol compared to Hepes MPS


A - Hepes MPS
B - Cyclohexanol
0.5mV
0.2ms

4.4 Discussion

As I have shown in Chapter 3, pesticide ingredients and their metabolic breakdown products have multiple effects on neuromuscular synaptic transmission. Therefore, the primary objective of this chapter was to carry out a more detailed and rigorous investigation of the mechanisms behind these alterations. As I have discussed in previous Chapters (1 & 3), the characteristic features of intermediate syndrome following organophosphorus pesticide poisoning in humans is the prolonged muscle weakness leading to muscle paralysis. However, I showed in Chapter 3 that acute exposure to pesticide ingredients and their metabolites led to prolonged nerve-evoked muscle contractions. Therefore, I felt it necessary to explore the possible mechanisms which affect the functional unit of muscle contraction and how this might contribute to muscle paralysis. At a cellular level, motor units comprise two main components, namely muscle fibres and the motor nerve terminals that innervate them. Therefore, I sought to examine first whether pesticide ingredients or their metabolic breakdown products change the passive membrane properties of the muscle fibres including input resistance, time constant and action potential threshold. I then turned my attention to properties of postsynaptic endplate currents, recorded under voltage clamp.

Technical challenges faced during this part of study may have affected the quality of my observed results. These include, damage of the muscle fibres and quality of the TEVC. Low resistance recording and current passing electrodes tend to damage the muscle fibre when impaled, producing an inadequate seal between the electrode tip and the muscle fibre membrane, thereby allowing leakage of the ion in and out of the membrane. This could have affected the measurements input resistance and the time constant, as well as magnitude and stability of endplate currents. Another potential caveat is that inadequate voltage clamp affects the amplitude of the synaptic current (low gain of the amplitude with larger escape of membrane voltage). On the other hand, minimum voltage escapes (higher gain of the amplifier) can lead to oscillations to the recorded current traces, which affect the time course of the waveform. Furthermore, the bathing medium temperature also affected the TEVC efficiency. Even though I replaced the bath solution often, heat generated from the surrounding equipment, including the microscope light source increased the bath medium temperature quickly. Therefore, in future using a perfusion system of chilled bathing medium may yield more reliable data.

Notwithstanding the reservations noted above, I did not see any alterations in the passive membrane properties of the muscle fibre with any of the pesticide ingredients or metabolites, suggesting that all the observed modulations of transmission I reported in Chapter 3 are due

to effects at the neuromuscular junction alone. Thus, I moved on to examine neuromuscular transmission in more detail by examining both post synaptic and presynaptic mechanisms, including neurotransmitter release upon evoked stimulation. I observed that metabolic breakdown products of the pesticide ingredients have additive or synergestic effects on the properties of the neuromuscular junction. Of interest, a preliminary recording in cyclohexanol treated preparations appears to show similar effects to vesamicol on neuromuscular transmission. These observations need further detailed examination in order to validate my findings.

4.4.1 Passive membrane properties are unaffected by the pesticide ingredients or their metabolic breakdown products.

In addition to the anticholinesterase effects displayed by the dimethoate and omethoate I hypothesised that cyclohexanone and cyclohexanol might change the passive membrane properties, which could result in modifications of synaptic potentials. I tested this hypothesis by examining input resistance and membrane time constant of the muscle fibres. The reduced excitability of axons in the presence of cyclohexanol suggested that perhaps this compound might have a general effect on reducing membrane resistance, producing "leakier" muscle fibres. However I did not observe any change in input resistance or membrane time constant with any of the treatments. I also hypothesized that muscle paralysis in intermediate syndrome could be due to reduced capacity to initiate action potentials. I examined this possibility by measuring at action potential threshold. However, neither threshold stimulating current nor thresholds potential were changed in the presence of pesticide ingredients or their metabolic breakdown products. Previously it was reported that some anticholinesterases change input resistance of muscle fibres (Kelly and Ferry, 1994), while others do not (Blaber, 1972). However, I could find no reports or evidence of solvent or its metabolites action on passive membrane properties. Therefore I concluded that the synaptic potential changes I observed are due to combinatorial effects of anticholinesterase activity and more direct action of all these ingredients on neuromuscular function.

4.4.2 Pesticide metabolites produce complex effects on the post synaptic NMJ

In order to understand the more complex effects of pesticide metabolites, described in the previous chapter, I set out to examine EPC characteristics with these compounds. The amplitude of the MEPC or EPC produced depend on the amount of ACh available to act and

the number of channels (nicotinic ACh receptors) being activated. Furthermore, the time course of the endplate current depends on the time course of the conductance change caused by each quantum of ACh in addition to the time course of the release of quanta (Gage and Armstrong, 1968). Omethoate produced slow but relatively similar peak amplitude MEPC compared to Hepes MPS, while cyclohexanol produced fast, but relatively smaller size MEPC compared to those recorded in Hepes MPS. However, EPC characteristics differ from MEPC in cyclohexanol, which produced larger responses compared to Hepes MPS. Omethoate produced slow EPC requiring a double exponential fit to the decay time. This suggests that the time course of the conductance change has two different components consistent with direct action of omethoate on receptors, changing their gating kinetics. Cyclohexanol also had similar effects on the requirements for exponential fit, its decay time also needing a double exponential fit, although cyclohexanol did not prolong the overall time course of the decay. It has been shown that anticholinesterases have concentration-dependent effects on the MEPC/EPC. Neostigmine, physostigmine and edrophonium at a concentration less than 10μM produced increases in EPC amplitude; while higher concentrations depressed the increment of EPC amplitude (Albuquerque et al., 1988). My results showing increased decay with omethoate confirming previous reports of anticholinesterase action on EPC decay (Takeuchi and Takeuchi, 1959; Gage and Armstrong, 1968; Kordas, 1972; Kordas et al., 1975). It was observed previously that the nicotinic non-competitive antagonist meproadifen interacts with the closed ionic channel of the ACh receptor in its resting and activated but non-conducting state, and only slightly affects the open conformation of the ionic channel (Maleque et al., 1982). There is also a possible mode of action of pyridostigmine independent of AChE inhibition (Pascuzzo et al., 1984). These authors speculated that pyridostimine blocks AChR thus producing non-conducting intermediates and changes in the conductance properties of the activated channels. This observation was confirmed by the direct action of pyridostigmine on the receptor complex (Akaike et al., 1984). They showed that pyridostigmine reduced channel conductance with unaltered channel life time. They also indicate that a higher concentration, of the drug (200μM) induced irregular waves of bursting activity during the initial phase of the application and, subsequently, significantly reduced the frequency of channel opening, thus indicating the pyridostigmine reacts with the ACh receptor, and both alone or in combination with ACh, induced an altered, desensitized species of the nicotinic AChR-ion channel complex. Moreover it was reported that physotigmine interact directly with AChR and blocks its open conformation (Kuba et al., 1974; Albuquerque et al., 1984; Albuquerque et al., 1985). It is also observed that higher concentrations (≥ 50μM) of neostigmine also interact directly with the endplate receptor

channel complex thus altering the gating kinetics and subsequently blocking the ACh receptor (Fiekers, 1985). Neostigmine and pyridostigmine act mainly as partial agonists, while physotigmine is mostly an inhibitor of the channel in the activated receptor conformation. These drugs bind to ACh recognition sites and to sites at the ionic channel as non-competitive blockers of the AChR.

Several studies have showed responses to conformational changes of AChR with anticholinesterase drugs. These include decreased channel conductance and prolonged channel life time (Akaike et al., 1984; Albuquerque et al., 1984; Pascuzzo et al., 1984). Also it is reported that direct interaction of physostigmine with nAChR blocks the ionic channel in its open conformation with a concomitant decrease of conductance and open time of the channel activation, while VX (nerve gas) only showed the shortening of open time with unaltered conductance (Albuquerque et al., 1985; Shaw et al., 1985).

These observations of receptor gating modulations with different anticholinesterase might contribute to the explanation of observed double exponential decay with omethoate in my experiments. However, there are few reports on the action of cyclohexanol with nAChR, except Aguayo and Albuquerque (1986), who demonstrated a double exponential decay function with 1-phenyl-4-piperidino-cyclohexanol treated preparations. Some reports suggest the cyclohexanol analogue vesamicol possess anticholinesterase activity on frog NMJ (Van der Kloot, 1986). However, cyclohexanol did not produced significant prolongation of MEPC/EPC decay in my experiments. Nevertheless further experiments such as ACh iontophoresis in isolated single muscle fibres and patch clamp recordings of single channels are needed to understand the action of cyclohexanol on the nAChR.

4.4.3 Modulation of transmitter release from presynaptic terminal is complex in the presence of pesticide components

When quantal content was calculated from EPPs I observed a reduction in omethoate and cyclohexanol treated preparations, either singly or in combination. But, quantal content was unaffected when I use EPCs for the calculations. Non-linear summation effects on the EPPs, might have affected the final calculations of quantal content. Moreover, the experiments in which the recorded EPPs were carried out using tibial nerve / FDB muscle preparations, while my EPC experiments were carried out on intercostal nerve / triangularis sterni muscle. Intriguingly, this observation was opposite to the effects observed in the EPP driven estimates. Hence, it may be necessary to examine further transmitter release from

presynaptic terminals in the presence of these compounds (pesticide parent and metabolic compounds).

Effects of anticholinesterases on evoked transmitter release have also been reported previously. Some studies have reported that evoked transmitter release with anticholinesterases shows a reduction in quantal content (Straughan, 1960; Potter, 1970; Laskowski and Dettbarn, 1975), while others reported there is no effect on quantal content (Blaber and Christ, 1967; Ferry and Marshall, 1971; Blaber, 1972).

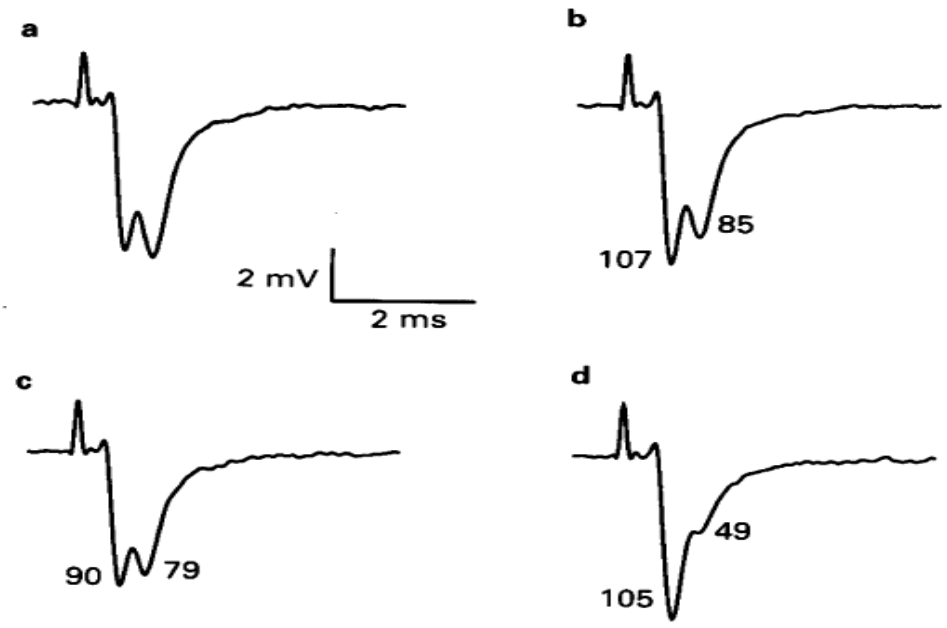
Quantal content (m) is determined by the number of quanta (n) released from the presynaptic terminal and the probability (p) of release of the quanta ($m=np$). Several factors including empty vesicles in the presynaptic terminal or changes in presynaptic currents could affect the probability of release thereby changing the quantal content in the presence of pesticide components. Another plausible hypothesis could be feedback mechanism from the accumulated ACh at synaptic cleft (due to anticholinesterase activity) exerted on the presynaptic terminal in order to minimise the amount of quanta release. It has been suggested that presynaptic nicotinic AChRs act as auto-receptors (Meir et al., 1999). This role was suggested by the evidence shown on effects of pharmacological modulation of these receptors at mammalian NMJ, showing these receptors have the ability to modulate transmitter release from the nerve terminal (Wessler et al., 1986; Wessler et al., 1992). Furthermore, some previous studies have also shown a reduction in quantal content with anticholinesterase treatment (Straughan, 1960; Potter, 1970; Laskowski and Dettbarn, 1975). Provan (1991) has shown the opposing effects on probability of transmitter release with different AChE inhibitors. They observed increase in m with increase p in tetrahydro-aminoacridine (an anticholinesterase drug prescribed for patients with Alzheimer disease), while physostigmine showed a decrease in m with a decrease in p. However neostigmine caused an increase m with unchanged p, suggesting different mechanism of action for changes in quantal content with different anticholinesterases. Even though there is some evidence for anticholinesterase effects on neurotransmitter release, lack of information regarding the solvent and their metabolites action on transmitter release makes it more difficult to understand the general sequelae; i.e. transmitter release from pesticide treated preparation.

4.4.4 Cyclohexanol may exert vesamicol-like effects

In addition to the anticholinesterase (dimethoate and omethoate) effects on NMJ transmission, the pesticide solvent (cyclohexanone) metabolite, cyclohexanol may have another, effect on neuromuscular transmission, reminiscent of the effects of vesamicol. (-)-Vesamicol is a reversible, non-competitive inhibitor of the transport of ACh into synaptic vesicles (Whitton et al., 1986). Searl et al (1991) showed that (-)-vesamicol has stimulation-dependent effects on reducing MEPC mean amplitude and changes in the distribution of MEPC amplitude due to inhibition of vesicular filling with ACh. Similarly, my preliminary experiments with cyclohexanol also produced stimulation sensititve reductions in MEPC amplitude. This vesamicol-like effect requires further investigation.

Examination of nerve terminal currents with cyclohexanol also showed a change in the characteristics of waveform compared to Hepes MPS. Previous reports on vesamicol action on perineural recordings also showed a inhibitory effects on nodal Na^+ -channels (fig 4.19) (Pemberton et al., 1992). Furthermore Naves & Van der Kloot (1996) reported that vesamicol action is more pronounced in the presence of anticholinesterase. Since I have seen more potent effects on modulation of NMJ synaptic transmission with the combination of omethoate and cyclohexanol together, the mechanism of action of cyclohexanol may be similar to that of vesamicol. Further detailed examination such as perineural recordings of presynaptic ion currents and detailed observation of pre stimulation and post stimulation MEPP distribution with cyclohexanol treated preparations is needed to confirm these observations.

Fig 4.19: Representative examples of nerve terminal cuurent waveforms recorded from a single impalement in the absence (a,b) and presence (c,d) of 100µM (-)-vesamicol. "Figure shows the first (a,c) and last (b,d) waveforms from a 6s period of 50Hz nerve stimulation. The numbers in (b), (c) and (d) give the amplitude of the two negative-going components of the waveform as a percentage of their amplitude in (a)." Figure and legends were adopted from Pemberton et al 1992.


a
2 mV
2 ms
b
107
85
c
90
79
d
105
49

Chapter 5: Use of an *ex-vivo* organ culture assay as a model of intermediate syndrome in pesticide poisoning

5.1 Introduction

In the previous two chapters, I characterized and explored the mechanisms by which pesticide components and their metabolites act on NMJ after acute exposure *in-vitro*. However, I did not see any neuromuscular transmission block and / or muscle paralysis in experiments utilising the pesticide components even after *in vitro* nerve-muscle preparations were treated with combinations of pesticide and its metabolites (Chapter 3 & 4) at concentrations similar to those present in IMS patients and the minipig model (Eddleston et al., 2012). This is puzzling but it suggests, perhaps, that in order for IMS to develop either other constituents of pesticide-poisoned plasma are required, or perhaps longer exposure to the components I investigated thus far is needed. Therefore, in this chapter I studied the effects of longer exposure to pesticide components and their metabolites in an attempt to mimic the conditions in which IMS develop. For this, I exploited the advantages of a relatively novel *ex-vivo* organ culture assay (Di Stefano et al., 2014; Brown et al., 2015). This comprised an isolated nerve-muscle preparation from FDB and lumbrical muscles maintained for up to 48 hrs in oxygenated MPS. These preparations undergo Wallerian degeneration of motor terminals and axons over this period, but the rate of this can be modified genetically and by administration of various compounds (Di Stefano et al., 2014; Brown et al., 2015).

Wallerian degeneration is a process which taken place after nerve injury, which the distal part of the axon, separated from the neuron's cell body, degenerates. This process occurs in both peripheral nervous system and central nervous system. It was first described by Augustus Waller in 1850, following severance of hypoglossal and glossopharyngeal nerves in frogs, cutting off the distal axons from their cell bodies. Since then, Wallerian degeneration has been studied in many aspects including neurodegenerative disorders in humans (Coleman and Freeman, 2010; Conforti et al., 2014). The discovery of *Wld*^{s} (Wallerian-degeneration slow) spontaneous mutant mice has benefitted our understanding of the mechanisms of Wallerian degeneration (Lunn et al., 1989; Conforti et al., 2014). It has also shown that there is a delay in synaptic degeneration about tenfold following axonal injury in homozygous *Wld*^{s} young adult mice (Gillingwater and Ribchester, 2003). This finding enables *Wld*^{s} mice to be used as a test bed for studying the mechanisms of Wallerian degeneration (Gillingwater et al., 2002).

Wallerian degeneration is also associated with organophosphorus toxicity. Organophosphorus induced delayed neuropathy (OPIDN) is a Wallerian type axonopathy

that occurs weeks to months after exposure of certain OP compounds (Abou-Donia, 2003). Wohlsein et al (2012) have also shown that spontaneous degenerative polienecephalomyelopathy in parathion intoxicated feeder pigs is associated with Wallerian degeneration of ventral rootlets and neurogenic muscle atrophy of limb muscles which is more similar to pathological features seen in equine and human motor neuron disease. In contrast Kawabuchi et al (1991) have shown non Wallerian type axonal degeneration of the neuromuscular synapse after single exposure to a sub-lethal dose of OP *in-vivo*. Even though IMS is a delayed muscle paralysis, availability of reports on morphological examination of neuromuscular junction during the morbidity is rare.

Since around 2000, mice transgenic for yellow fluorescent protein (YFP), (a variant of the jellyfish green fluorescent protein (GFP) in motor nerves have become available (Feng et al., 2000), labelling both nerve terminal and axons. These were subsequently cross bred with *Wld*^{s} mice to yield double mutations with fluorescent axons and motor terminals that were also protected by *Wld*^{s} expression, and this has become more valuable resource for the studying morphological evidence of the degenerative process (Beirowski et al., 2004; Beirowski et al., 2005; Wong et al., 2009). These mice were originally generated using a modified thy 1 gene to promote expression of YFP (Caroni, 1997; Feng et al., 2000; Lichtman and Sanes, 2003).

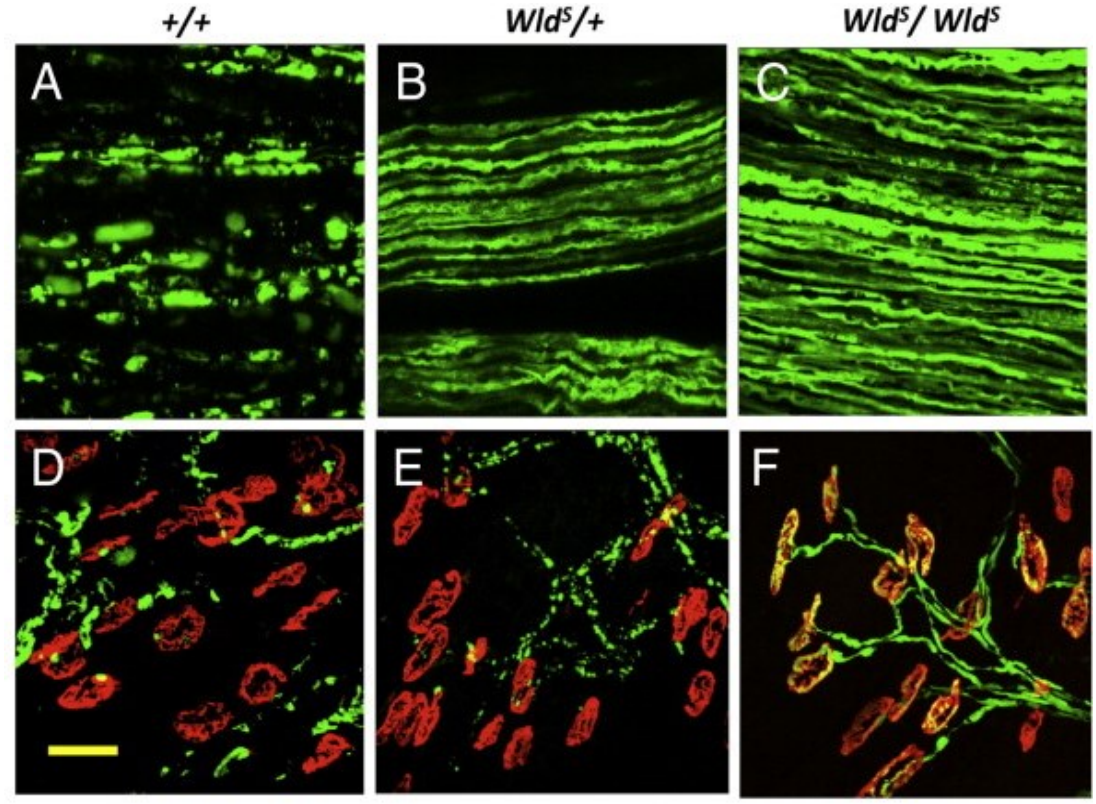
Previous research in our laboratory has shown that the time course of synaptic degeneration in vivo is delayed in *Wld*^{S} mice (Ribchester et al., 1995; Gillingwater et al., 2002; Bridge et al., 2009; Wong et al., 2009; Brown et al., 2015). These studies used thy1.2 YFP16 *Wld*^{s} mice and demonstrated that homozygous *Wld*^{s} mice have a longer time course of synaptic degeneration compared to heterozygous mice or wild type mice (fig 5.1, 5.2). Recently our lab has developed an organotypic explant assay (*ex-vivo*), using homozygous thy1.2 YFP16 *Wld*^{s} mice to test for modulators of synaptic degeneration (Di Stefano et al., 2014; Brown et al., 2015). This novel organ culture system was established using tibial nerve - FDB muscle preparations and demonstrated the difference of the *Wld*^{s} phenotype from wild-type 24-48hrs after overnight incubation at 32°C (Brown et al., 2015). This organ-culture system allows us not only to test the modulators of synaptic degeneration, but also to evaluate the effects and mechanisms of long term exposure to different compounds active on neuromuscular transmission, thus identifying and screening for efficacy of potential novel treatments.

Therefore, the aims of my experiments in this chapter were first to explore the effects of 24hr exposure of pesticide ingredient and their metabolites in the NMJ, and to evaluate whether

this might reproduce effects of pesticide ingestion that give rise to IMS in humans. Next I utilize a paradigm for activity dependence test for these components on NMJ transmission. Finally, I asked whether synaptic degeneration might be triggered or enhanced by the extended exposure to pesticide ingredients and their metabolites.

My results suggest that the isolated *ex-vivo* *Wld*^{S} mouse nerve-muscle preparation may constitute a novel and innovative method for evaluating pesticide toxicity and its modification.

Fig 5.1: Motor nerve terminals are protected from Wallerian degeneration in *Wld*^{s} mice. Conventional confocal microscopic images of distal tibial nerve axons (A–C) and neuromuscular junctions in deep lumbrical muscles (DL; D–F) three days after ipsilateral section of the sciatic nerve. Axons and nerve terminals are labelled via transgenic expression of YFP. Postsynaptic acetylcholine receptors were labelled with TRITC-α-bungarotoxin to visualize motor endplates. Axons and motor nerve terminals degenerate rapidly in wild-type (+/+; A,D). Axons are protected in *Wld*^{s} heterozygotes (B,E), which express about half the level of mutant protein compared with homozygotes, but motor nerve terminals are not. Both axons and nerve terminals are protected in *Wld*^{s} homozygotes (C,F). The time course of synaptic degeneration (G) was measured by scoring the percentage of all endplates in whole mounts of DL muscles (about 250 fibres per muscle) that were 'vacant': that is, with no overlying YFP-positive terminal (as in D,E). Each point represents mean data from up to four lumbrical muscles from three to four mice (total number of axotomized lumbrical muscles examined D—33; E—56; F—29). Calibration bar: 50 μm. (Fig and legend from Wong et al 2009).


+/+
Wld^S/+
Wld^S/ Wld^S
A
B
C
D
E
F

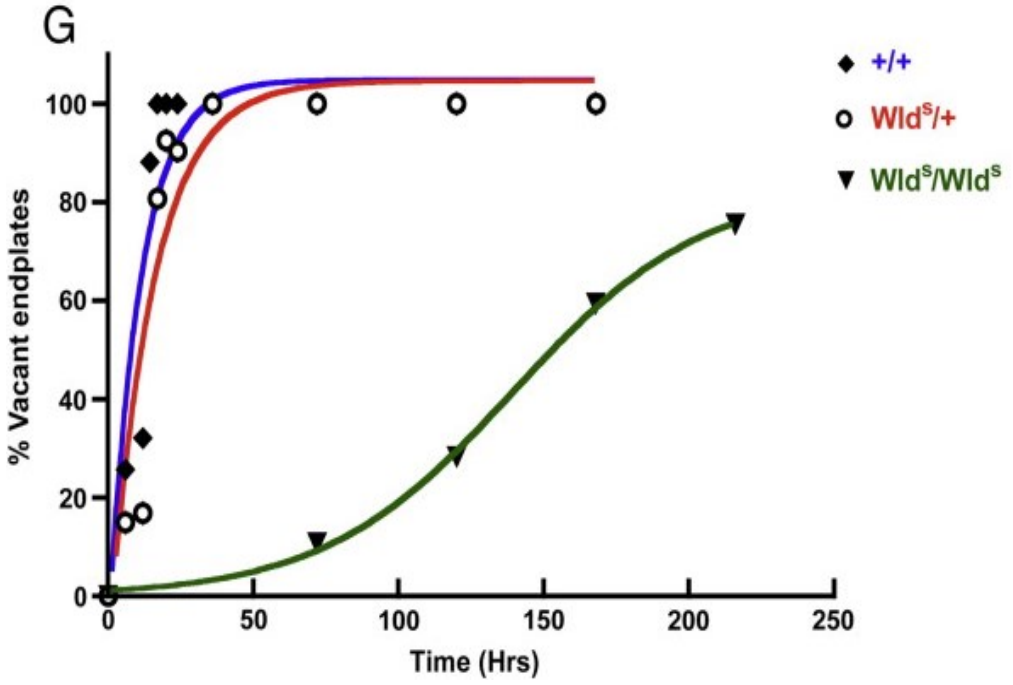
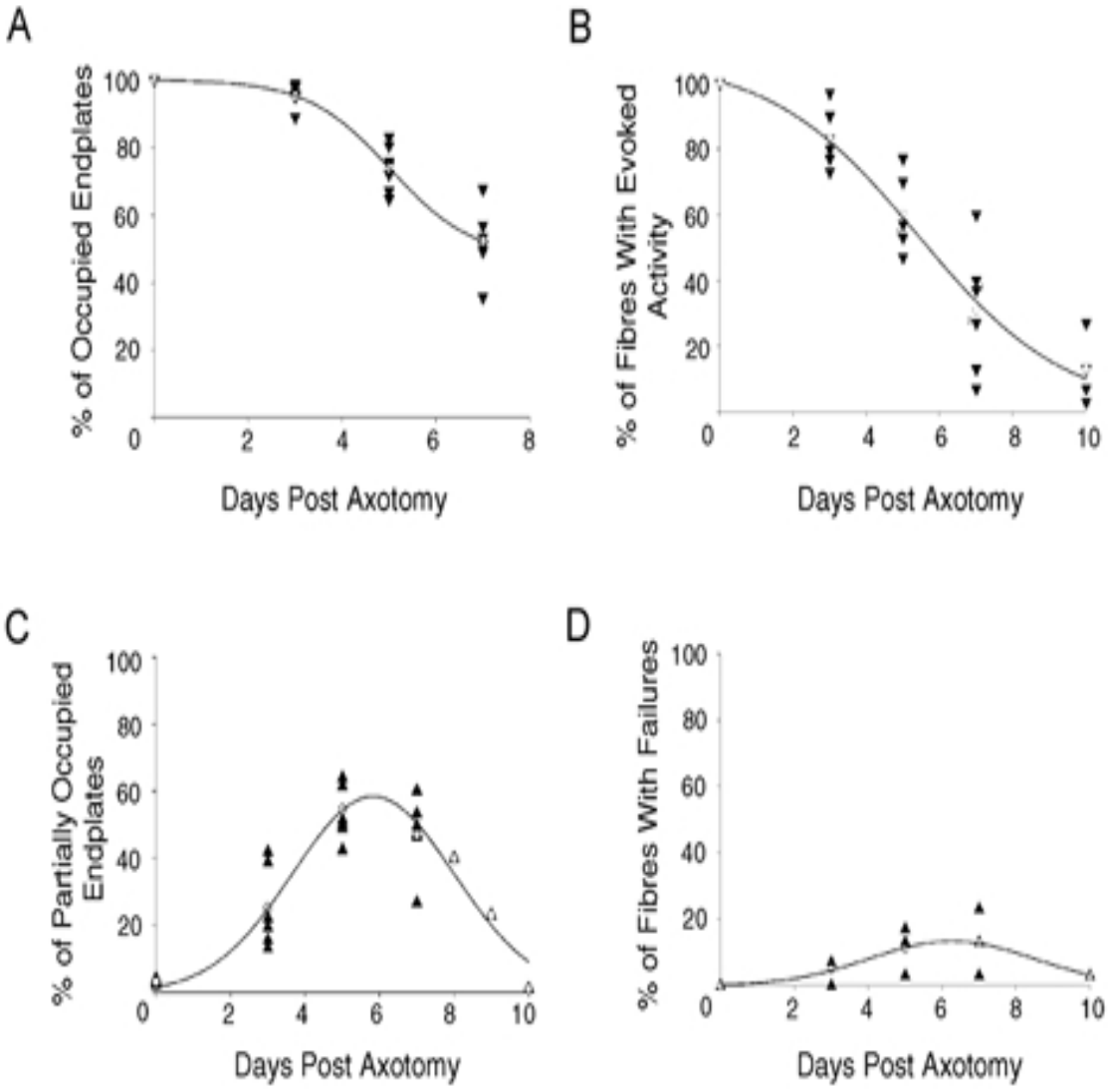

G
% Vacant endplates
Time (Hrs)
+/+
Wld^S/+
Wld^S/Wld^S
0
20
40
60
80
100
0
50
100
150
200
250

Fig 5.2: Time course of synapse withdrawal in 2-month-old *Wld*^{s} mice. *A*, morphological data showing the time course of nerve terminal withdrawal in 2-month-old *Wld*^{s} (homozygous) mice FDB muscles following axotomy. *B*, electrophysiological measurements of the time course of nerve terminal loss in 2-month-old *Wld*^{s} mice FDB muscles following axotomy. *C*, morphological analysis of the incidence of endplate partial occupancy in 2-month-old *Wld*^{s} mice FDB muscles following axotomy. *D*, electrophysiological analysis of the incidence of failures in response to nerve stimulation in 2-month-old *Wld*^{s} mice FDB muscle fibres following axotomy. For all panels, ▲ and ▼ represent data points from individual muscles and ○ indicates the mean value calculated at each time point. (Graphs and legend adopted from Gillingwater et al 2002).


A
% of Occupied Endplates
100
80
60
40
20
0
2
4
6
8
Days Post Axotomy
B
% of Fibres With Evoked Activity
100
80
60
40
20
0
2
4
6
8
10
Days Post Axotomy
C
% of Partially Occupied Endplates
100
80
60
40
20
0
2
4
6
8
10
Days Post Axotomy
D
% of Fibres With Failures
100
80
60
40
20
0
2
4
6
8
10
Days Post Axotomy

5.2 Materials and methods

The following methods are specific to this chapter. For general methods and chemicals, refer to Chapter 2.

Base line recordings for all the experiments were carried out in bicarbonate buffered MPS at room temperature unless otherwise stated on specific occasions.

5.2.1 Animals

In these experiments I primarily used C57 *Wld*^{s} mice. In some of the experiments I also used homozygous thy1.2 YFP16 *Wld*^{s} mice (*Wld*^{s} mice cross bred to double homozygous thy 1.2 YFP 16 (commercially available line from Jackson laboratories) (Feng et al., 2000; Wong et al., 2009). The genotyping of these mice was carried out routinely for YFP expression by checking for fluorescent axons in ear punches (removed upon weaning), under a fluorescence microscope. All the *Wld*^{s} mice used in this chapter were aged between 6 to 8 weeks in order to avoid the age dependent decline in *Wld*^{s} phenotypic characteristics (Gillingwater et al., 2002).

5.2.2 Ex-vivo organ culture assay

The rational for the approach in this Chapter is that axons and motor nerve terminal degenerate very slowly in *Wld*^{s} mice after axotomy *in-vivo* (Gillingwater and Ribchester, 2001) The phenotype is partially reproduced in *ex-vivo*, when muscles are cultured over night at 32° (Di Stefano et al., 2014; Brown et al., 2015).

Bicarbonate buffered Mammalian Physiological saline (MPS: 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.4mM $NaHCO_3$, 5.6mM Glucose) was prepared at room temperature and equilibrated with continuous bubbling of medical gas (5% CO_2 and 95% O_2) in order to set its pH value with in physiological range (7.2 – 7.4). Mice (*Wld*^{s} or thy1.2YFP16:*Wld*^{S}) were sacrificed and amputated hind limbs were pinned in a Sylgard-lined Petri dish containing MPS, for dissection. The tibial nerve along with the FDB and deep lumbrical muscles attached together were dissected. The nerve-muscle preparation was then pinned on dental wax (Polysciense Europe, Inc., Germany) through muscle proximal and distal tendons. The preparation was then transferred to a sterile 30ml universal tube containing MPS ± drug treatment for overnight incubation. The tube was placed in a water bath at 32^{o} C and connected to continuous supply of medical gas (5% CO_2 and 95% O_2

mixture) and bubbled for 24hrs, prior to commencing both physiological and morphological analysis. The whole apparatus was covered in aluminium foil in order to prevent evaporation of liquid and damage by the direct sunlight. After 24 hrs, preparations were transferred to freshly prepared MPS ± drug treatment before commencing further analysis. Physiological analysis was carried out in two steps: first scoring the number of innervated muscle fibres, followed by acute intense stimulation for one hour.Scoring of the muscle fibres was then repeated after stimulation to ascertain any use-dependent deterioration of synaptic function with or without drug treatment.

5.2.2.1 Sampling protocol for scoring innervated muscle fibres

Scoring of innervated muscle fibres were performed (ex-vivo organ culture assay FDB nerve muscle preparation) prior to μ-CTX pre-treatment. Estimate of innervated muscle fibres were calculated from 10 – 20 randomly selected muscle fibres. Innervated muscle fibres were defined as those giving rise to spontaneous MEPP and evoked responses (EPP ± action potential). These were subcategorized as a) fully functional / innervated muscle fibres (presence of MEPP and EPP ± action potential) and b) partially functional / paralysed muscle fibres (spontaneous MEPP only). Denervated muscle fibres were defined as those producing neither MEPP nor evoked response (EPP ± action potential) upon stimulation. This categorization represents a low stringency test for effects of pesticide ingredients and their metabolites on neuromuscular transmission and function.

The preparations were then subjected to acute stimulation for one hour, followed by the previous step of scoring for innervated fibres (20). The protocol adopted for acute stimulation comprised trains of four stimuli at 2Hz frequency, repeated every 10 seconds for one hour.

5.2.3 Fluorescence microscope measurements

Visualization of the nerve terminals and motor endplates was accomplished based on the YFP fluorescence of axons and TRITC α-bungarotoxin labelling of all junctional receptors. Both 10X and 20X objectives of the fluorescence microscope were used to visualise the NMJs. Images were captured using a Hamamatsu Orca-ER camera coupled to an Apple Mac G5 computer, and processed using Openlab software (Improvision, UK). NMJs were classified as fully occupied, partially occupied or vacant, depending on the extent of occupancy of TRITC- α-bungarotoxin labelled endplates by the overlying YFP labelled axon. When there was no YFP labelled axon coverage on endplates (vacant), it was

considered a denervated muscle fibre (method for semiquantitative analysing of synaptic degeneration).

5.3 Results

5.3.1 24hrs exposure of pesticide and its metabolites reduced the number of innervated muscle fibres.

The primary objective of this set of experiments was to identify whether protracted exposure to pesticide ingredients and their metabolites in different combinations affects the functional capacity of neuromuscular junctions. Reduction in the number of functional muscle fibres would also produce muscle paralysis; so my attempt was to examine whether the *ex-vivo* preparation mimicked IMS in any fashion.

The data revealed that the percentage of responsive muscle fibres (giving rise to MEPP or EPP ±AP) after 24hrs incubation with DOCC (dimethoate1mM, omethoate 100µM, cyclohexanone 1mM, cyclohexanol 5mM) was significantly reduced compared to incubation in MPS alone (MPS: 100 ± 0%, dimethoate + cyclohexanone; 93.33 ± 7.63%, omethoate + cyclohexanol; 80 ± 8.66%, omethoate + cyclohexanone + cyclohexanol; 80 ± 18.02%, dimethoate, omethoate, cyclohexanol; 76.66 ± 7.63%, DOCC; 77 ± 5.70%, mean ± SD, $P < 0.05$, ANOVA, "F = 17.84") (fig 5.3, 5.4). Combinations of dimethoate and cyclohexanone preparations acted similar to control MPS, showing no significant reduction in responsive muscle fibres after 24hrs exposure to the treatments. However, all the other combinations of pesticide ingredients and their metabolites showed a significant reduction in responsive muscle fibres compared to control MPS.

To characterize the effects in more detail responsive muscle fibres were sub categorised, depending on their ability to produce evoked responses upon stimulation. The data showed that there was a significant increase in fibres showing only spontaneous MEPPs in the presence of DOCC and dimethoate + omethoate + cyclohexanol treated preparation. Percentages were compared with bicarbonate MPS was: MPS: 8.75 ± 2.31%, dimethoate + cyclohexanone; 12 ± 15.71%, omethoate + cyclohexanol; 11.66 ± 2.88%, omethoate + cyclohexanone + cyclohexanol; 21.66 ± 2.88%, dimethoate, omethoate, cyclohexanol; 56.66± 18.92%, DOCC; 39 ± 21.33%, mean ± SD, $P < 0.05$, ANOVA, "F = 8.64" (Fig 5.5). Reduction of evoked responses in preparations treated with dimethoate + omethoate + cyclohexanol was similar to preparations treated with DOCC suggesting that cyclohexanone

does not have considerable effects on evoked transmission. Collectively, the data also show that prolonged exposure to DOCC had a strong effect on evoked transmission.

These data support the suggestion that muscle fibre paralysis due to inability to produce an evoked synaptic response might contribute to IMS. Since the pesticide ingredients alone did not affect the responsive muscle fibres, the data suggest that the most important components to affect muscle fibre innervation are the pesticide metabolites, i.e. omethoate and cyclohexanol. Collectively, these data support the hypothesis that longer term exposure to pesticide ingredients and their metabolites are required to blocks NMJ synaptic transmission.

Fig 5.3: Example traces of action potential / EPP with different treatments. A: Bicarbonate MPS, B; dimethoate (1mM) and cyclohexanone (1mM), C; omethoate (100µM) and cyclohexanol (5mM), D; DOCC. Note that the prolonged decay time with combination of omethoate and cyclohexanol and DOCC treated preparations.

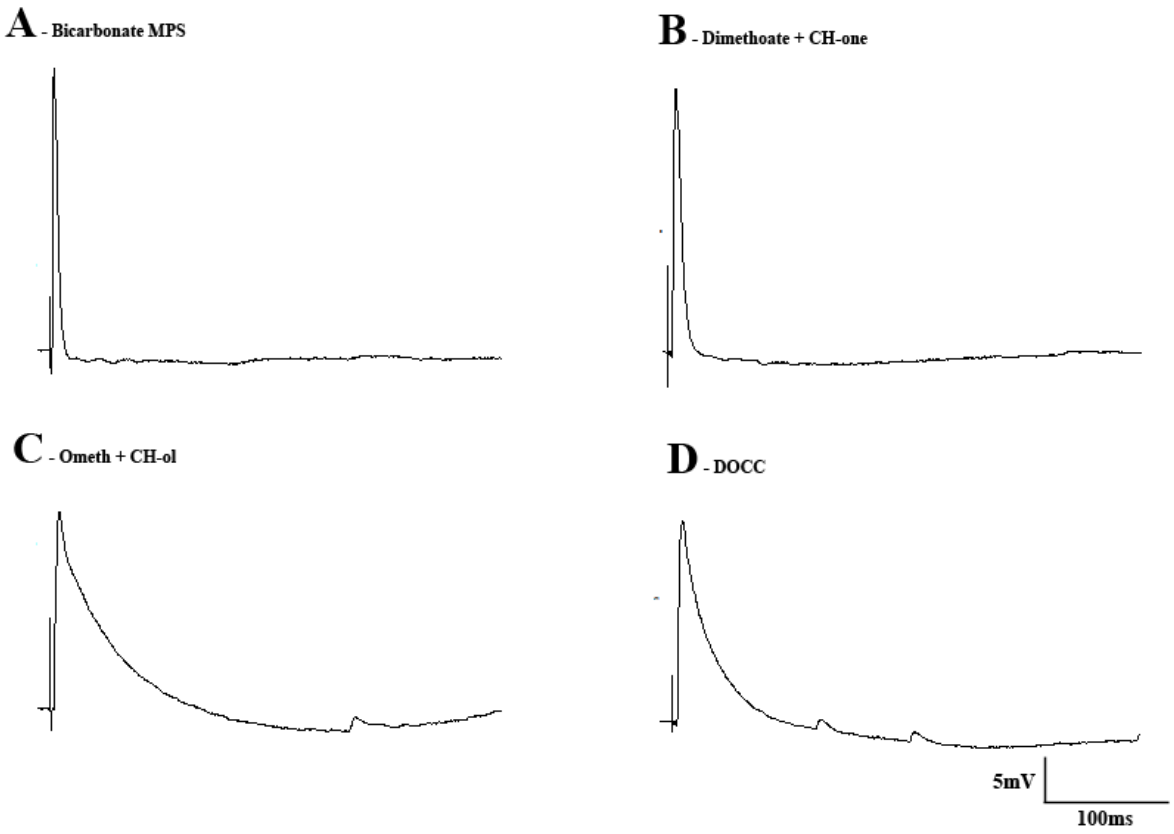

A - Bicarbonate MPS
B - Dimethoate + CH-one
C - Ometh + CH-ol
D - DOCC
5mV
100ms

Fig 5.4: Percentage of responsive muscle fibres (MEPP ± EPP/AP). Note that the reduction in responsive muscle fibres when omethoate and cyclohexanol combined together. However, long term exposure of pesticide ingredients does not reduce the responsive muscle fibres. Each bar represents mean ± SEM [n (No of muscles): MPS = 17, dimethoate + cycloxexanone = 3, omethoate + cyclohexanol = 3, omethoate + cyclohexanone + cyclohexanol = 3, dimethoate + omethoate + cyclohexanol = 3, DOCC = 3] ($p < 0.05$, **ANOVA, Bonferroni post test were compared with Bicarbonate MPS / control**).

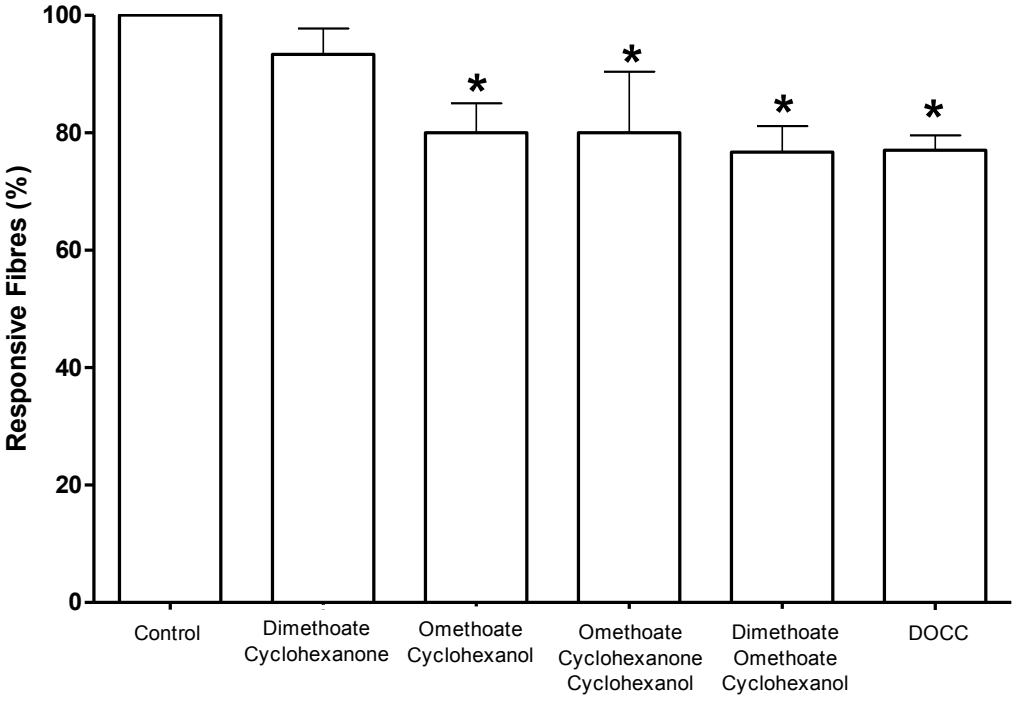
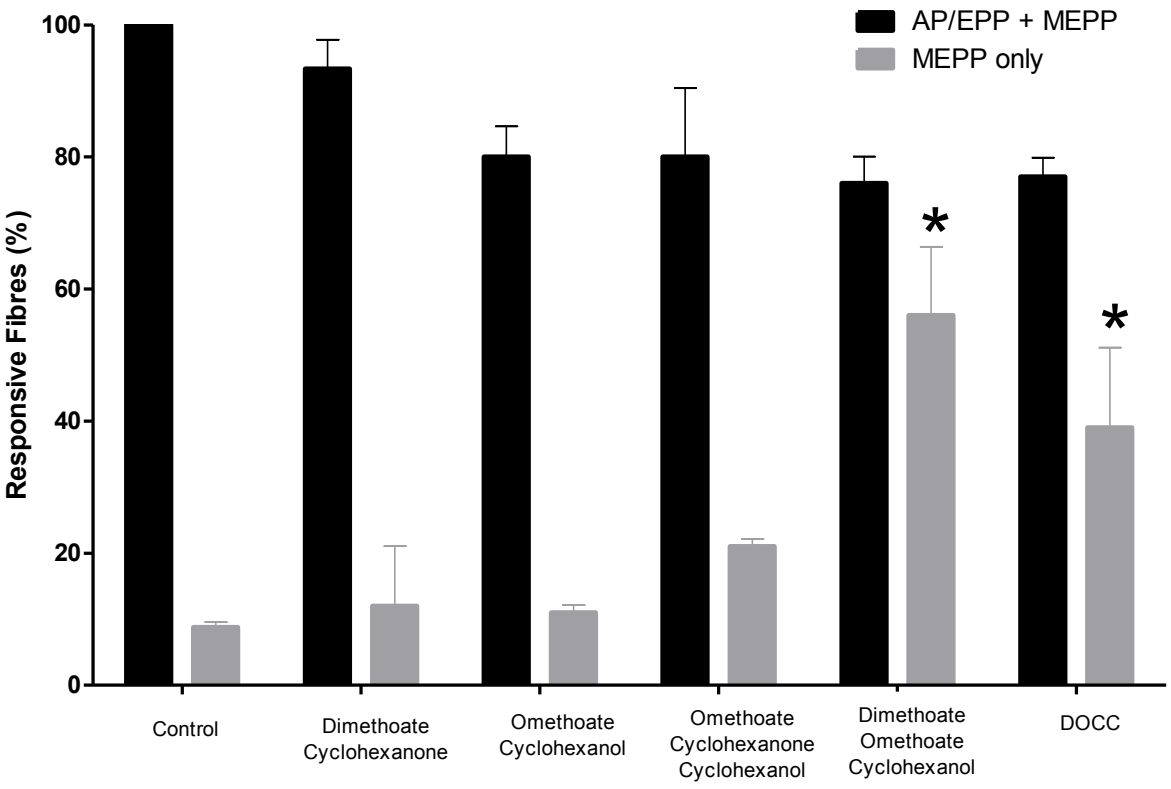

Responsive Fibres (%)
100
80
60
40
20
0
*
*
*
*
Control
Dimethoate
Cyclohexanone
Omethoate
Cyclohexanol
Omethoate
Cyclohexanone
Cyclohexanol
Dimethoate
Omethoate
Cyclohexanol
DOCC

Fig 5.5: Percentage of responsive muscle fibres expressing either MEPP ± EPP/AP or MEPP only. Note the significant increase in MEPP only responsive fibres with DOCC and dimethoate + omethoate + cyclohexanol treated preparations, showing that these components possibly affect evoked transmission. Each bar represents mean ± SEM [n (No of muscles): MPS = 17, dimethoate + cycloxexanone = 3, omethoate + cyclohexanol = 3, omethoate + cyclohexanone + cyclohexanol = 3, dimethoate + omethoate + cyclohexanol = 3, DOCC = 3] ($p < 0.05$, **ANOVA, Bonferroni post test were compared with Bicarbonate MPS / control**).


AP/EPP + MEPP
MEPP only
Responsive Fibres (%)
100
80
60
40
20
0
*
*
Control
Dimethoate
Cyclohexanone
Omethoate
Cyclohexanol
Omethoate
Cyclohexanone
Cyclohexanol
Dimethoate
Omethoate
Cyclohexanol
DOCC

5.3.2 Acute stimulation after long term exposure of pesticide ingredients and their metabolites enhances loss of transmission.

Next I explored whether the failure of NMJ synaptic transmission after 24hr exposure to pesticide components and their metabolites might be further exacerbated by activity. In normal healthy individuals, the most active muscle groups at rest are the respiratory muscles (due to continuous breathing) and neck muscles (to balance the body posture). In pesticide poisoned patients, due to cardiorespiratory shock (hypoxemia and hypotension) even higher demand are placed upon the respiratory muscles during the acute cholinergic episode. Therefore I hypothesised that increasing activity along with the prolonged exposure to pesticide ingredients and their metabolites might also be a contributing factor for muscle paralysis present in IMS. To test this I stimulated the nerve-muscle preparations after overnight culture with treatment, using trains of four stimuli (TOF) (2Hz) every 10 seconds for one hour. I then reassayed the amount of functional innervation.

The data revealed that preparations treated with DOCC produced a significant reduction in responsive fibres after one hour of acute stimulation (table 5.1, fig 5.6). Interestingly, however, none of the other treatments produced additional use-dependent block.

These data suggests that there is apparently activity dependent modulation of synaptic transmission in DOCC treated preparations.

Fig 5.6: Percentage or responsive muscle fibres (MEPP ± EPP/AP) in before and after acute stimulation. Note that the reduction in responsive muscle fibres upon acute stimulation with combination of dimethoate, omethoate, cyclohexanol and DOCC treated preparations compared to rest of the treatment groups. Each bar represents mean ± SEM (n = 3 muscles per each treatment group) ($p < 0.05$, **Two-way, repeated measures, ANOVA, Bonferroni post test**).

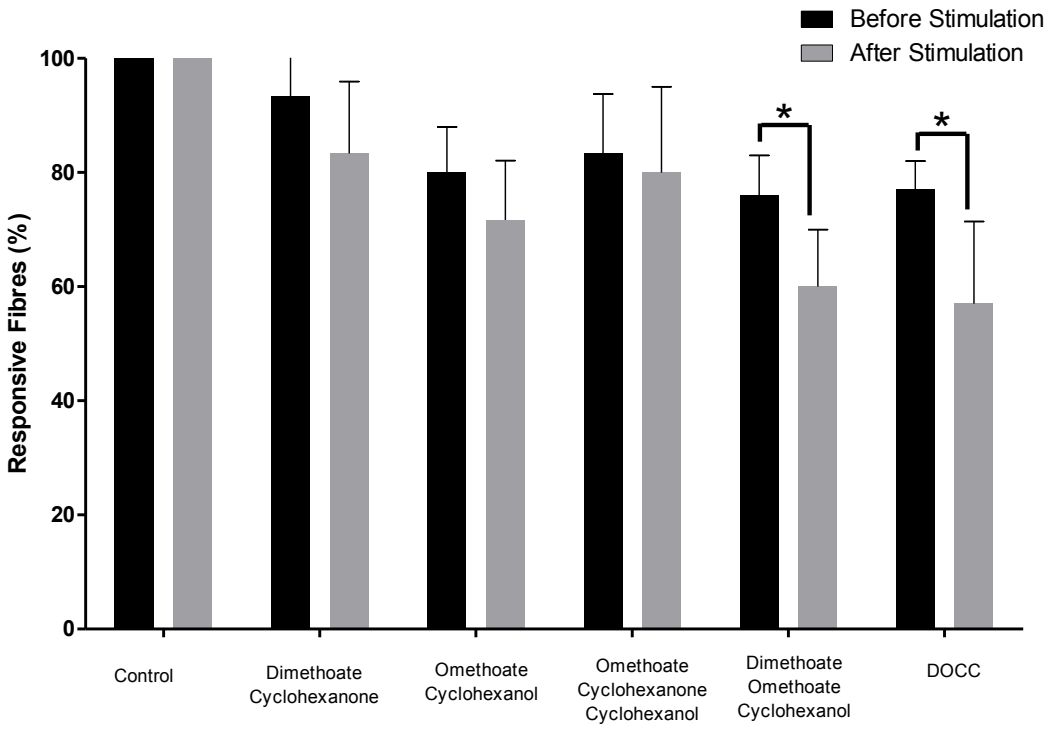

Before Stimulation
After Stimulation
Responsive Fibres (%)
100
80
60
40
20
0
*
*
Control
Dimethoate
Cyclohexanone
Omethoate
Cyclohexanol
Omethoate
Cyclohexanone
Cyclohexanol
Dimethoate
Omethoate
Cyclohexanol
DOCC

Table 5.1: **Summary of statistical analysis of effects on acute stimulation with pesticide ingredients and their metabiolites**. Statistical test; twoway analysis of variance. Post test; Bonferroni multiple comparison test (n = 3 muscles per each treatment group).

Before Stimulation vs After Stimulation				
Row Factor	Before Stimulation	After Stimulation	Difference	95% CI of diff.
Control	100	100	0	-5.840 to 5.840
Dimethoate + Cyclohexanone	93.33	83.33	-10	-24.30 to 4.304
Omethoate + Cyclohexanol	80	71.67	-8.333	-22.64 to 5.971
Ometh+CH-one+CH-ol	83.33	80	-3.333	-17.64 to 10.97
Dimeth +Ometh+CH-ol	76	60	-16	-30.30 to -1.696
DOCC	77	57	-20	-34.30 to -5.696
Row Factor	Difference	t	P value	Summary
Control	0	0	P > 0.05	ns
Dimethoate + Cyclohexanone	-10	1.915	P > 0.05	ns
Ometh+CH-ol	-8.333	1.596	P > 0.05	ns
Ometh+CH-one+CH-ol	-3.333	0.6383	P > 0.05	ns
Dimeth +Ometh+CH-ol	-16	3.064	P < 0.05	*
DOCC	-20	3.829	P < 0.01	**

5.3.3 Long term exposure to pesticide and metabolites causes synaptic degeneration

Finally I asked whether DOCC might have caused paralysis in the overnight cultures by enhancing synaptic degeneration.

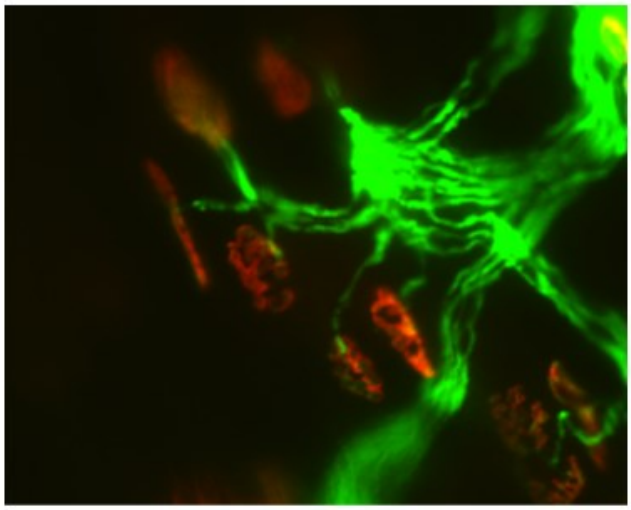
Lumbrical muscles from YFP16 *Wld*^{s} mice were counterstained with TRITC-α-bungarotoxin, to label post synaptic ACh receptors after 24 hour exposure to DOCC. These muscles showed signs of enhanced Wallerian degeneration (fig 5.7). Control muscles showed no signs of degeneration. Subjective and semi-quantitative analysis (number of vacant endplates) of the treated preparation showed increased synaptic degeneration with the combination of omethoate and cyclohexanol and with the DOCC treated-preparations compared to control MPS (table 5.2). However, these observations need further investigation to clarify whether the observed synaptic degeneration in combination of omethoate and cyclohexanol and DOCC treated preparations can account for the physiological evidence of synaptic dysfunction.

Table 5.2: Semi quantitative analysis (number of vacant endplates) of synaptic degeneration, after 24hrs exposure of pesticide ingredients and their metabiolites in nervemuscle preparations. Note that the enhanced degeneration with pesticide metabolites (omethoate and cyclohexanol) and DOCC treated preparations. "+" sign indicate the amount of degeneration (vacant endplates / no overlying nerve terminal) present in the sample.

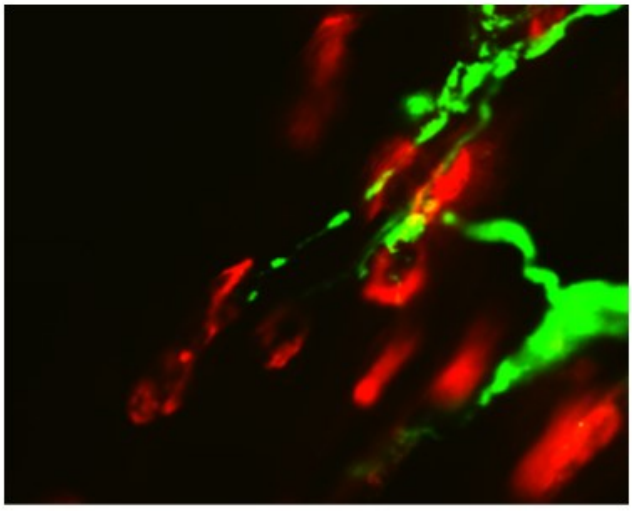
Treatment	**Degeneration**
Control MPS	+
Dimethoate and cyclohexanone	+
Omethoate and cyclohexanol	+++
DOCC	+++

Fig 5.7: Motor nerve terminal undergoes degeneration after long term exposure to DOCC. Fluorescence microscopic images of neuromuscular junctions in deep lumbrical muscles after 24 hrs exposure to either bicarbonate buffered MPS alone (A) or DOCC (B). Axons and nerve terminals are labelled via transgenic expression of YFP (green). Postsynaptic acetylcholine receptors were labelled with TRITC-α-bungarotoxin (red) to visualize motor endplates. Note that intramuscular axons are intact and all the endplates in control (A) samples are occupied with an overlying, YFP-positive terminal, while almost all the axons in the DOCC treated preparation (B) are fragmented and the endplates are vacant (that is, with no overlying YFP-positive terminal) in this field of view.

A



B



50µM

5.4 Discussion

The primary aim of this Chapter was to establish and study the effects on neuromuscular transmission of longer term exposure (24 ± 1 Hrs) to the concentrations of pesticide and metabolites found in poisoned plasma. My objective was to establish whether an *ex-vivo* preparation, developed in our laboratory, could be used to emulate the IMS of human pesticide-poisoned patients. Furthermore, I wanted to test whether there was an activity dependent modulation of synaptic transmission in the presence of pesticide components and to investigate any other evidence for synaptic degeneration with longer exposure to these compounds.

I observed that direct exposure for 24hrs to pesticide and metabolites produced a significant reduction in innervated muscle fibres (presence of either spontaneous or evoked transmission). More surprisingly, among those innervated muscle fibres, there were a significant proportion of paralysed muscle fibres (spontaneous transmission only). These data may therefore provide some insight towards the muscle paralysis present in human pesticide poisoned patients. Furthermore I also observed activity-dependent modulation of synaptic transmission in preparations treated with pesticide and their metabolites (DOCC). This suggests that increased activity in affected muscle groups might contributed to the muscle paralysis observed in patients with IMS. Finally, there was some evidence of synaptic degeneration after long term exposure (24 ± 1 Hrs) to high concentration of DOCC suggesting involvement of several mechanisms, not all of which are apparent in human IMS.

Further refinement of the experimental protocol may be necessary to simulate IMS. In human pesticide poisoned patients (and minipigs model of OP pesticide toxicity), when the toxins are ingested these compounds first undergo absorption from the gut and intestinal epithelium. Next, these parent compounds undergo metabolic conversion. Some of these compounds (both parent and metabolites) are excreted via urine. Therefore, it takes time (approximately about 3 – 5 hrs) to accumulate these compounds in the plasma and tissue. However, in the experiments described in Chapters 3 & 4, I introduced the active compounds immediately into the bathing medium, rather than gradually and producing change in the relative concentrations of the compounds over time. Therefore, in future the experiments in the present chapter could be further refined by introduction of these compounds gradually into the nerve muscle preparation (such as use of perfusion system over the 24hrs time period) which might help us to understand the time course of this muscle paralysis. Moreover, I have used an acute stimulation protocol to evaluate the role of activity on use-dependent block in IMS. However, it will be more rational to apply stimulation in

continuous bursts during the 24hrs period. This would more accurately simulate the pattern of respiration or attempted muscle use *in-vivo*. Finally, ex-vivo preparations of respiratory related muscle such as phrenic nerve - diaphragm or intercostal nerve - trangularis sterni muscle might be more appropriate for exploring the mechanisms of this respiratory muscle paralysis in human patients.

However, in principle this ex-vivo culture system could be used as an efficient and inexpensive screening tool to test for possible treatments for NMJ synaptic transmission failure in IMS. For example, it will be possible to test for protective effects of blocking the post synaptic receptors using nicotinic receptor antagonist (eg; rocuronium) along with the DOCC, and to examine whether reduction in repetitive activity will rescue the NMJ transmission in the long run. This proposal is based on the partial success that has been observed already in minipigs pre-treated with rocuronium (Eddleston, personal communication / unpublished) and a clinical trial underway in Sri Lanka using rocuronium as a potential prophylactic agent against onset of IMS.

5.4.1 Long term (24 ± 1 hrs) exposure of pesticide ingredients and their metabolites has greater effects on NMJ synaptic transmission

Despite the observed effects on NMJ synaptic transmission including increasing in decay time of synaptic potentials and currents, after acute exposure of pesticide and metabolites either singly or in combination, as I described in my previous results chapters (Chapter 3 & 4), I did not observe NMJ synaptic transmission block as described in intermediate syndrome patients, except when I applied pesticide poisoned plasma from pigs. Therefore, I hypothesised that in order to produce muscle paralysis / NMJ synaptic transmission block, longer exposure to these compounds would be required. I tested this hypothesis by examining NMJ synaptic function after long term exposure of those compounds using ex-vivo culture assay. The observation of reduction in responsive muscle fibres suggests the mechanisms might be involved in muscle paralysis intermediate syndrome patients. Reduction in muscle fibres able to produce evoked responses suggests possible involvement of many mechanisms (excitation, release, action and excitation-secretion coupling). Since it was still possible to observe MEPPs, this shows that spontaneous transmission is still intact. Therefore, I suggest that mechanisms involved in evoked transmission such as excitation of the presynaptic terminal or secretion of ACh from presynaptic terminals are affected; thus preventing EPPs / APs which normally lead to muscle action potentials and muscle contraction. From the previous results (Chapter 4), I demonstrated that passive muscle

membrane properties were not affected by short term exposure to these components. Therefore, this suggests that further examination of NMJ synaptic transmission is needed to locate the failure point of excitation and secretion mechanisms of NMJ synaptic transmission.

Involvement of activity dependent modulation in synaptic transmission has been studied for many decades. It has been shown that fast and slow muscle NMJs display marked plasticity by being able to adapt important release characteristics to the impulse patterns imposed on them (Reid et al., 2003). Furthermore, some studies have also reported that remarkable plasticity and adaptive responses to the chronic absence of AChE, which has direct consequence of the functioning of the neuromuscular junction (Girard et al., 2005). Therefore I wanted to examine how activity along with the pesticide its metabolites modulate the synaptic plasticity. My experiments showed that acutely increased activity is harmful to the NMJ synaptic transmission in the presence of DOCC, producing further reduction of innervated muscle fibres compared to preparations wtih unstimulated muscle fibres but exposed to DOCC.

Finally, the data also suggested that DOCC induced synaptic degeneration. However, further quantitative analysis of the effect is required to clarify where enhanced degeneration explains the physiological findings. Although there is no direct evidence on synaptic degeneration on IMS patients or animal models of OP poisoning, there are few reports of limited muscle necrosis in both cases. Human IMS patients have shown myopathic changes (both muscle biopsies and post mortem samples) including focal necrosis of the muscles (De Reuck and Willems, 1975; De Wilde et al., 1991; John et al., 2003); however, they did not report any abnormalities in peripheral nerves (De Wilde et al., 1991). Similarly, animal studies of OP pesticide toxicity also have reported increase muscle necrosis after exposure to OP agents including pesticide and nerve gases (Salpeter et al., 1979; Hughes et al., 1991)). These previous observations suggest that morphological changes in cellular architecture of NMJ may occur in IMS. Furthermore, it has also been shown previously, that OPIDN involves Wallerian degeneration (Abou-Donia, 2003).

Collectively, from the data and the previous reports, I suggest that the involvement of synaptic degeneration in IMS along with the activity dependent transmission block with longer term exposure of pesticide ingredient and its metabolites might be a plausible explanation for the neuromuscular transmission block. The ex-vivo paradigm I utilised here

may turn out to be a useful model for studying IMS, with the potential to screen and identify possible new treatments and management options for patients with IMS.

Chapter 6: General Discussion

The data presented in this thesis may have an important bearing on the treatment of the delayed synaptic transmission failure at the neuromuscular junction in organophosphorus pesticide poisoning.

Though IMS has been documented for decades, the underling mechanism is still unclear. The hypothesized mechanism of action for IMS includes: overstimulation of nicotinic acetylcholine receptors (nAChRs); down regulation of post synaptic nAChRs; and nAChR conformational changes with prolonged depolarization (Senanayake and Karalliedde, 1987; De Bleecker, 1995). Developing a better understanding of this delayed neuromuscular transmission with OP pesticide poisoning may benefit patients by discovering effective treatment and therefore saving many lives in the future.

Effects of AChE inhibition on neuromuscular transmission had been studied in both functional and cellular aspect since mid-20th century (Brown et al., 1936; Brown, 1937; Eccles and O'Connor W, 1941; Fatt and Katz, 1951; Del Castillo and Katz, 1954; Senanayake and Karalliedde, 1987). However, experimental evidence on mechanisms of delayed, muscle paralysis or neuromuscular transmission failure due to AChE inhibition is rare. The components of OP pesticide include active ingredients (OP compound), solvent (organic) and a surfactant. Moreover, when ingested these components undergo metabolism, mainly in the gastric epithelium and liver. Even though anticholinesterase activity on synaptic transmission at NMJ is well documented, the effects of solvents on synaptic transmission at the NMJ were yet to have been explored.

A previous study involving intensive care of a minipig model of OP poisoning has suggested that both anticholinesterase and solvent may be necessary for the development of the neuromuscular transmission failure (Eddleston et al., 2012). Therefore, I hypothesised that pesticide toxicity is not entirely due to acetylcholinesterase inhibition, but to combinatorial effects of both OP and the solvent present in the pesticide. Hence, the aim of my project was first to investigate whether the acute syndrome is caused only by the anticholinesterase effects of pesticide at the neuromuscular junction or whether metabolic conversion of the parent compounds has more potent, additive toxic effects at the NMJ. I focused on one particular pesticide; Dimethoate Ec. Secondly, I investigated mechanisms involved in possible neuromuscular transmission failure with pesticide ingredients and their metabolites. Finally, to model the neuromuscular transmission failure in IMS and to verify whether it might be due to the persistent combined effects of metabolites of pesticide ingredients (omethoate and cyclohexanol) on neuromuscular transmission, I studied the effects of

pesticide ingredients and their metabolites on isolated nerve-muscle preparations maintained for 24 hrs *ex vivo*.

In Chapter 3, I provided evidence for effects of synaptic transmission at NMJ with components of pesticide either alone or in combination. Intracellular endplate potential recordings from nerve-muscle preparations treated with minipig plasma obtained from pigs treated with either commercial pesticide, dimethoate alone, or saline showed increase in decay time of the EPP with pesticide plasma or dimethoate plasma compared to saline plasma or Hepes MPS. Preparations treated with neostigmine as a positive control showed decay times of both MEPPs and EPPs that, as expected, were significantly increased compared to Hepes MPS (Blaber and Christ, 1967; Katz and Miledi, 1973; Kordas, 1977; Miledi et al., 1984). However, the decay time facilitation comparing minipig pesticide plasma or dimethoate plasma was not statistically different. Interestingly, the latency for prolongation of the decay time with plasma (pesticide or dimethoate) was longer compared to neostigmine. This suggests complex mechanisms may underly the response to plasma, such as: a) anticholinesterase activity present in plasma may have been lower than expected, b) some of the molecules of anticholinesterases may be bound to other molecules in the plasma, inactivating them or slowly releasing. Interestingly, pesticide treated plasma also produced a significant increase in MEPP frequency compared to the other treatment groups suggesting increase in spontaneous release activity of neurotransmitter from presynaptic terminals. Changes in MEPP frequency with AChE inhibition had been observed previously including increase (Blaber and Christ, 1967; Laskowski and Dettbarn, 1975; Bois et al., 1980) and decrease (Duncan and Publicover, 1979) of spontaneous activity. However, neither dimethoate plasma nor neostigmine produced such an increase in MEPP frequency suggesting this may be an effect of both anticholinesterase and solvent/metabolite present in the pesticide plasma. More importantly, reversible evoked synaptic transmission block was observed with pesticide plasma treated samples, suggesting complexity of the evoked transmission with the presence of both anticholinesterase and solvent. There were previous observations of either depolarizing block (Nastuk and Alexander, 1954; Katz and Thesleff, 1957a; Blaber and Christ, 1967) or irreversible transmission block (Laskowski and Dettbarn, 1979), however neither the positive control (neostigmine) nor dimethoate plasma produced synaptic transmission block suggesting the observed transmission block with pesticide plasma is a consequence of multiple effects including, effect of AChE inhibition and presence of molecules other than OP in the pesticide plasma.

Treatment of nerve muscle preparation with pure ingredients of pesticide components and their metabolites showed greater effects with metabolites of pesticide compared to its parent components. It has been widely accepted that thiophosphorus OP compounds need bio-activation inside the body to become more potent (Foxenberg et al., 2007; Eddleston and Clark, 2010). The cumulative dose response of pesticide ingredients indicated increased potency of drug action with metabolite (omethoate) compared to its parent (dimethoate) compound. More interestingly, the solvent metabolite cyclohexanol also showed more pronounced effects on synaptic transmission at NMJ compared to its parent component cyclohexanone.

Synergistic / additive effects on prolongation of synaptic transmission were observed when omethoate and cyclohexanol (metabolites) were combined. This was a significant finding as concentration of omethoate (100μM) used in these experiment produced > 99% AChE block, suggesting cyclohexanol contributed to the synaptic transmission prolongation by a mechanism other than AChE inhibition. The synergistic effects were supported by the functional assay of synaptic transmission at NMJ: muscle contraction. High frequency tetanic contraction with omethoate and cyclohexanol produced sustained after contraction with prolonged relaxation compared to rest of the treatments.

Furthermore, high frequency repetitive stimulation revealed short term synaptic transmission depression with some evidence suggesting desensitization when preparations were treated with combination of all parent components and metabolites (DOCC). These findings suggested involvement of activity in producing synaptic transmission impairment in the presence of these components. Increase activity dependent synaptic transmission modification has been studied previously (Dorlochter et al., 1991; Fahim, 1997); however the use-dependent transmission impairment at NMJ with pesticide components is yet to be fully explained.

Importantly, I did not observe a neuromuscular transmission block with a combination of dimethoate, omethoate, cyclohexanone and cyclohexanol (DOCC) *in vitro* (both intracellular recordings and muscle contraction), similar to reversible evoked transmission block noted with the pesticide plasma experiments. However, Eddleston et al (2012) have reported transmission block in minipigs either with treatment of pesticide or combination of dimethoate and cyclohexanone. This suggests that in order to develop synaptic transmission block, not only these components are necessary, but also other mechanisms are involved

(e.g. either additional metabolites, or prolonged action of these components and continuous activity at NMJ).

In Chapter 4, I examined the possible mechanisms of actions in relation to the synaptic transmission failure at the NMJ. First I measured the effects on passive membrane properties with pesticide components either alone or in combination. Some early reports have shown that changes in input resistance of the muscle fibres with AChE inhibition (Kelly and Ferry, 1994), however my results did not show any alterations in any passive membrane properties with anticholinesterase alone, similar to Blaber et al (1972), or in combination with solvent. This suggests that these compounds do not produce leakier muscle fibres.

Having excluded the possible alterations in passive membrane properties, I then measured characteristics of the underlying currents affected by omethoate and cyclohexanol, either alone or in combination. Omethoate produced prolongation of the decay time, consistent with the evidence for anticholinesterase activity (Takeuchi and Takeuchi, 1959; Gage and Armstrong, 1968; Kordas, 1972; Kordas et al., 1975). Interestingly, omethoate induced an EPC decay time that required a double exponential fit, suggesting two mechanisms of actions for the prolongation of decay. Previous reports of effects of anticholinesterase activity on EPCs also showed evidence for complex mechanisms, perhaps consistent with a direct action of these compounds on the nAChR, changing the gating kinetics of the channels (Kordas, 1972; Kuba et al., 1974; Kordas et al., 1975; Akaike et al., 1984; Albuquerque et al., 1984; Albuquerque et al., 1985). Furthermore, some reports provided evidence for direct blocking of the ACh receptor with neostigmine (Fiekers, 1985). This raises the possibility that omethoate might also have direct effects on the AChRs, which might be responsible for blocking the channel complex. Even though cyclohexanol did not prolong EPC decay substantially, it also conferred a double exponential decay suggesting a complex mechanism of action. Among the few documented pieces of evidence available on effects of cyclohexanol on synaptic transmission at NMJ, it was shown that double exponential decay function in EPC with 1-phenyl-4-piperidino-cyclohexanol treated preparation. Furthermore, the cyclohexanol derivative vesamicol also possesses anticholinesterase activity at frog NMJ (Van der Kloot, 1986). Remarkably, in the presence of both omethoate and cyclohexanol more than two exponentials were required to fit the EPC decay. This intriguing result further suggests that complex, independent effects of these compounds on AChRs. This could perhaps be investigated by performing ACh iontophoresis using isolated single muscle fibres (Bekoff and Betz, 1977b; Gillespie and Ribchester, 1988); or and patch clamp recordings

from single channels in order to measure the direct effects of these compounds on the AChR gating.

Having examined the possible post synaptic effects, I then examined the presynaptic characteristics with these components. First I estimated the influence on transmitter release from presynaptic terminals in the presence of pesticide components. Estimates of quantal content using EPPs suggested a significant reduction in transmitter release with the combination of metabolites or all four components together. Studies have reported alterations in transmitter release with AChE inhibition showing reduction in quantal content (Straughan, 1960; Potter, 1970; Laskowski and Dettbarn, 1975), while others reported there is no effect on evoked transmitter release (Blaber and Christ, 1967; Ferry and Marshall, 1971; Blaber, 1972). However, the use of EPPs to estimate quantal content has disadvantages as the amplitude of the EPPs can vary due to the change in driving force and non-linear summation. Therefore, as a more robust test I used EPCs to estimate quantal content. I obtained results with treatments that were not statistically different from the control. Hence, further experiments are needed to determine whether the effects of cyclohexanol on quantal content in FDB are a consequence of differences in the properties of motor nerve terminals of FDB compared with TS, or some other consequence of experimental recording conditions.

The observed reduction in MEPC amplitude with cyclohexanol treated preparations drew my attention towards whether these preparations behave as vesamicol, a non-competitive inhibitor of the transport of ACh into synaptic vesicles (Whitton et al., 1986). My observation of stimulation dependent reduction in MEPC amplitude demonstrated vesamicol-like effects (Searl et al., 1991) with cyclohexanol. Previous reports of vesamicol action on perineural recordings also showed inhibitory effects on nodal Na^+ -channels (Pemberton et al., 1992). The preliminary data from nerve terminal currents recorded with cyclohexanol treated preparations also showed some evidence of alterations. However, further experiments are required to confirm these findings, such as perineural recordings and evaluation of pre and post stimulation MEPP distribution in cyclohexanol treated preparations.

In Chapters 3 &4, I have tried to understand and explain the effects of acute exposure of pesticide compounds on synaptic transmission, thereby exposing the mechanisms involved. However, IMS is a delayed syndrome. In my third results chapter I have tried to model the neuromuscular transmission failure after chronic exposure to pesticide components, thus

exploring any similarities and mode of action to IMS in an *ex-vivo* model. 24hrs of chronic exposure to DOCC produced a significant reduction in innervated muscle fibres. Among these innervated muscle fibres, a significant proportion were paralysed, as they did not produce evoked responses. EPPs recordings obtained following high frequency repetitive stimulation in the presence of DOCC also showed short term synaptic depression within the cycle and desensitization in consecutive cycles (Chapter 3), demonstrating possible activity dependent effects on synaptic transmission. Therefore, I wanted to explore the effects of acute stimulation after chronic exposure to pesticide components. The data showed an activity-dependent reduction in innervated muscle fibres in DOCC treated preparations. Although activity-dependent modulation of synaptic transmission at NMJ is quiet debatable (Dorlochter et al., 1991; Fahim, 1997), the data I obtained suggest that increasing activity in the presence of DOCC is detrimental to reliable transmission.

The relatively small reduction I observed in innervated muscle fibres with both chronic exposure and upon acute activation in the presence of DOCC raises the question whether these findings are of significance for functional aspect of muscle contraction. For instance, ALS patients show muscle weakness after they have lost about 60% of the functional motor units (Krarup, 2011). In my experiments, I found that 60% of the fibres are still innervated after chronic exposure to DOCC, suggesting muscles should still be competent to contract. However, ALS is a progressive degenerative disorder and compensatory mechanisms such as collateral sprouting and muscle fibre hypertrophy contribute to the normal muscle contraction during early stages of the disease until they start showing clinical symptoms. In patients acutely poisoned with pesticide, there is only very limited time to activate compensatory mechanisms for reliable synaptic transmission in the event of loss of innervated muscle fibres. Therefore, acute loss of up to 40% loss of transmitting NMJs might plausibly explain the underlying signs and symptoms of IMS.

There was some evidence for synaptic degeneration in DOCC treated preparations, suggesting involvement of more complex mechanism in the presence of these compounds. There are some evidence on involvement of myopathy in IMS patients (De Reuck and Willems, 1975; De Wilde et al., 1991; John et al., 2003). Furthermore, experimental study of sarin exposure in mice also showed degeneration responses with collateral sprouting 2- 3 days after in-vivo treatment (Kawabuchi et al., 1989; Kawabuchi et al., 1991). However, on the other hand, intensive care minipigs model of OP toxicity does not provide sufficient evidence for possible degeneration effects after 12hrs from poisoning (Eddleston & Harris, personal communication). It will be more beneficial to further investigate any possible

degeneration effects along with the neurophysiological evaluation (*in-vivo and in-vitro*) in pesticide toxicity using an animal model, such as intensive care mouse model of OP toxicity (Dissanayake, MSc thesis 2010). Lack of evidence on neuropathic changes during the course of IMS in humans does not exclude the possible degenerative effects cause by these pesticide components. Future use of the techniques such as microendoscopy (Brown et al, 2014) in IMS patients may be able to detect any evidence of degeneration.

Future experiments should be directed along two main paths. First, further understanding of the additive or synergistic actions of omethoate and cyclohexanol, and how they may contribute to development of IMS. Second, is to develop therapeutic regime that can be utilized to prevent development of IMS and to enhance the recovery of patients from the respiratory paralysis, thereby reducing the probability of IMS related morbidities and mortalities.

In order to understand how omethoate and cyclohexanol augment the development of IMS, first it may be necessary to understand whether and how these components change the gating kinetics of AChRs. This could be attempted using patch clamp recording from single channels. Furthermore, it will be useful to know whether these components stimulate desensitization of the AChRs. This could be tested using ACh ionophoresis in the presence of the treatments. Changes in AChRs density could also be tested in the presence of these components in order to test the possibility of receptor internalization in IMS. Moreover, in order to identify the presynaptic alterations, changes in presynaptic ionic currents in the presence of these components could be evaluated using perineural recordings. Quantal analysis under low Ca^{2+} extracellular medium along with the treatment could help in identifying the role of extracellular Ca^{2+}. Furthermore in order to understand, whether there is any feedback regulation of transmitter release by presynaptic AChRs in the presence of these components, AChRs antagonists could be used to block these receptors.

Finally, the ex-vivo assay described in Chapter 4 could be used to test for both protective and therapeutic treatments for IMS. Protective mechanisms such as blocking of AChRs in order to prevent over stimulation could be tested using nAChRs antagonists. Since there is some evidence of protective action of neuromuscular blocking agents in data obtained from the pig model of OP toxicity, it may be possible to using this ex-vivo assay to identify any therapeutic window for commencement of treatment and understanding the underlying mechanism of action.

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